Actions of appetite regulating peptides on supraoptic nucleus (SON) oxytocin neurones

by

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PhD by Research

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Declaration

This thesis entitled, **"Actions of appetite regulating peptides on supraoptic nucleus (SON) oxytocin neurones"** submitted for the degree of **PhD by Research** in the field of Biomedical Sciences to the University of Edinburgh, has been composed by the candidate, **Sathya Velmurugan** (née Sathya Arunachalam), and the work is original and has not been submitted for any other degree of professional qualification. The assistance and help received during the course of investigation have been fully acknowledged.

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ABSTRACT

Oxytocin has established roles in parturition and lactation, but can also be released in response to non-reproductive stimuli, such as hyperosmolarity and stress. As a majority of appetite regulating peptides activate the hypothalamo-pituitary-adrenal stress axis, and oxytocin is also a stress hormone in the rat, it was hypothesized that the oxytocin system in the neurohypophysial axis could be a target for appetite-regulating peptides of central and peripheral origin. The effects of central administration of neuropeptide Y (NPY; a central orexigenic peptide and a central and peripheral neurotransmitter co-released with noradrenaline; n=5 rats) and systemic administration of secretin (a peripheral gut peptide belonging to the family of brain-gut peptides; n=26) and leptin (a peripheral anorexigenic peptide from adipose tissue; n=23) on the electrical activity of SON oxytocin neurones *in vivo* were studied in urethane-anaesthetized female rats with extracellular recording. Effects were compared with the excitatory responses to cholecystokinin (CCK; a peripheral anorexigenic gut peptide; n=45). Influences of fasting and pregnancy and effects of these peptides on the activity of SON vasopressin neurones were also studied. Results: (1) All the central and peripheral appetite peptides tested increased the electrical activity of SON oxytocin neurones. (a) NPY: Basal firing rate of 3.5 ± 1.05 (mean ± s.e.m) spikes/s was increased by 1 ± 0.45 spikes/s 1min after NPY (basal vs 0-10min post-NPY: P=0.03, paired t-test; n=5). (b) Secretin: Basal rate of 4.1 ± 0.4 spikes/s was increased by 1.7 ± 0.2 spikes/s 2.5min after secretin (basal vs 0-10min post-secretin: P<0.001, paired t-test; n=26). (c) Leptin: Basal rate of 3.4 ± 0.4 spikes/s was increased by 0.4 ± 0.08 spikes/s 1.5min after leptin (basal vs 0-10min post-leptin: P=0.01, paired t-test; n=23). (d) CCK: Basal rate of 3.6 ± 0.3 spikes/s was increased by 1.1 ± 0.15 spikes/s 1min after CCK (basal vs 0-10min post-CCK: P<0.001, Wilcoxon signed rank test; n=45). (2) Secretin induced excitatory responses were greater than to other peptides (P<0.001, Kruskal-Wallis one-way ANOVA on ranks). (3) Secretin dose-dependently increased SON oxytocin neurone electrical activity and peripheral oxytocin release in anaesthetized rats. (4) Intracerebroventricular infusion and microdialysis studies with benoxathian (α1 adrenergic antagonist) revealed that secretin-induced excitation of SON oxytocin and vasopressin neurones involves central excitatory noradrenergic pathways. (5) Fasting for 18h did not alter the excitation of SON oxytocin neurones induced by secretin, CCK and leptin. (6) The pathway leading to excitation of oxytocin neurones by CCK was not influenced by prior leptin administration. (7) SON oxytocin neurones were responsive to leptin during late pregnancy. (8) NPY-induced excitation of oxytocin neurones was intact in anaesthetised late pregnant rats, contrasting with attenuated oxytocin secretory responses observed previously in conscious rats. (9) Systemic NPY excited SON oxytocin neurones. (10) Systemic CCK administration either inhibited (77%) or did not affect (23%) SON vasopressin neurones, while leptin had no significant effect, and responses to secretin were predominantly excitatory (67%). Systemic NPY inhibited vasopressin neurones, but central NPY was ineffective. Conclusion: Appetite peptides target SON oxytocin neurones. Postprandially released secretin and leptin might, like CCK, induce peripheral oxytocin release, so as to regulate water and electrolyte homeostasis, which is inevitably disturbed during feeding. Any central release of oxytocin induced by these peptides, might regulate feeding behaviour and satiety. Oxytocin neurone excitation induced by NPY may be relevant during stress responses.

ABBREVIATIONS

A1	A1 cell group in the brain stem (VLM)
A2	A2 cell group in the brain stem (NTS)
aCSF	Artificial cerebrospinal fluid
ACTH	Adrenocorticotropic hormone
AgRP	Agouti related peptide
ANOVA	Analysis of variance
ANP	Atrial natriuretic factor
AP	Area postrema
AQ	Activity quotient
ARC	Arcuate nucleus
AVP	Arginine vasopressin
Bar	Barrington nucleus
BBB	Blood brain barrier
BBC	British Broadcasting Corporation
BNST	Bed nucleus of stria terminalis
C1	C1 cell group
cAMP	Cyclic adenosine monophosphate
CART	Cocaine amphetamine regulatory transcript
CCK	Cholecystokinin
CCK	Cholecystokinin gene
CCK-8	Cholecystokinin octapeptide
CCK_AR	Cholecystokinin A receptor (CCK_1R)
CCK_BR	Cholecystokinin B receptor (CCK_2R)
CeA	Central amygdala
c-fos	An immediate early gene
cGMP	Cyclic guanosine monophosphate
CNS	Central nervous system
CPM	Count per minute
CRH	Corticotropin releasing hormone
CVO	Circumventricular organ
DHA	Dorsal hypothalamic area
DHS	Developmental hyperserotonemia
DMH	Dorsomedial hypothalamus
DMNV	Dorsal motor nucleus of the vagus
ENS	Enteric nervous system
Fos	An immediate early gene protein
GABA	Gamma – aminobutyric acid
GHRH	Growth hormone releasing hormone
GI	Gastrointestinal

GIP	Gastric inhibitory polypeptide
GLP-1	Glucagon-like peptide 1
GLP-2	Glucagon-like peptide 2
GnRH	Gonadotrophin releasing hormone
GPCR	G protein coupled receptor
HPA	Hypothalamo pituitary adrenal
i.c.v	Intracerebroventricular
i.p	Intraperitoneal
i.v	Intravenous
I-cells	Intestinal mucosal epithelial cells secreting cholecystokinin
IL-1β	Interleukin – 1 beta
ir	Immunoreactive
JAK-2	Janus kinase - 2
KiSS-1	A melanoma suppressor gene peptide
LC	Locus coeruleus
LDCV	Large dense core vesicles
LHA	Lateral hypothalamic area
MC4-R	Melanocortin 4 receptor
ME	Median eminence
mPOA	Medial preoptic area
mPVN	Magnocellular division of paraventricular nucleus
mRNA	Messenger ribonucleic acid
NA	Noradrenaline or Noradrenergic
NE	Norepinephrine
NHS	National Health Service
NO	Nitric oxide
NPY	Neuropeptide Y
NTS	Nucleus tractus solitarii
Ob-R	Leptin receptor
^{o}C	Degree Celsius
PACAP	Pituitary adenylate cyclase activating peptide
PBel	Lateral parabrachial nucleus
PFH	Perifornical hypothalamus
PHA	Posterior hypothalamic area
PHI	Peptide Histidine Isoleucine
PHM	Peptide Histidine Methionine
POA	Preoptic area
POMC	Proopiomelanocortin
PP	Pancreatic polypeptide
pPVN	Parvocellular division of paraventricular nucleus

pSTAT3	Phosphorylated signal transducer and activator of transcription 3
PVN	Paraventricular nucleus
PYY	Peptide YY
RIA	Radioimmunoassay
SC	Subcoeruleus nucleus
S-cells	Intestinal mucosal epithelial cells secreting secretin
SCN	Suprachiasmatic nucleus
SEM	Standard error of mean
SON	Supraoptic nucleus
SSV	Small synaptic vesicles
STAT3	Signal transducer and activator of transcription 3
STAT5	Signal transducer and activator of transcription 5
TH	Tyrosine hydroxylase
TRH	Thyrotropin releasing hormone
V1a/1b, V2	Vasopressin receptors
VIP	Vasoactive intestinal peptide
VLM	Ventrolateral Medulla
vmARC	Ventromedial arcuate
VMN	Ventromedial nucleus
vmPOA	Ventromedial preoptic area
WHO	World Health Organisation
YR	Neuropeptide Y receptor
α-MSH	Alpha melanocyte stimulating hormone

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CHAPTER I

INTRODUCTION

Appetite control encompasses the regulation of food intake, energy balance, adiposity and body weight. In recent years it has become clear that a range of peptide hormones or neuropeptides (together termed 'appetite peptides'), in the circulation or released in the brain, are essential elements of the systems controlling appetite. They act in or on complex neural networks in the brain. This thesis addresses the question of the possible roles of oxytocin in these networks by investigating responsiveness of oxytocin neurones to peptides that are well-established or putative appetite regulators.

1.1. Current obesity crisis necessitates understanding appetite regulation

Despite day-to-day fluctuations in the amount of food consumed, the body weight remains almost constant in adults. When the energy expenditure equals energy intake, the body is in a state of energy balance. There are short-term and long-term signals regulating this balance. However, if these signals are altered and the energy balance is disturbed, in such a way that intake exceeds expenditure, it results in weight gain potentially leading to obesity, or expenditure exceeds the intake resulting in cachexia.

The Association for the Study of Obesity[1] proclaims that the number of obese people is rising rapidly throughout the world, making obesity one of the most rapidly developing public health problems. The World Health Organisation (WHO)[2] describes the problem of obesity as a "worldwide epidemic". According to WHO, around 1 billion people worldwide are overweight[3], of which 300 million are clinically obese. The prevalence of obesity has increased by 75% worldwide since 1980 (Flegal, 2005).

The USA has a particularly high prevalence of obesity. On average, over one third of the adult population are obese, rising to more than 50% in some ethnic subgroups in the USA. Although the UK lags behind the USA, the rate of change is very similar to the USA (Source: Association for the Study of Obesity[4]). Almost 1 in 4 adults in England are currently obese. More than 12 million adults and one million children in England will be

[1] http://www.aso.org.uk/mlw/files/scale%20of%20the%20problem.pdf
[2] http://www.who.int/dietphysicalactivity/publications/facts/obesity/en/

[3] The WHO defines "overweight" as a body mass index (BMI) equal to or more than 25 and "obesity" as a BMI equal to or more than 30. BMI is defined as the weight in kilograms divided by the square of the height in meters (kg/m^2).

[4] http://www.aso.org.uk/mlw/files/scale%20of%20the%20problem.pdf

obese by 2010, according to the figures published by the Department of Health[5]. If it is carried on, by 2050, 9 in 10 adults will be overweight or obese.

Overweight and obese people cost the UK economy £7 billion in treatment, reduced productivity, loss of earnings and benefits. By 2050, this figure could be almost half the NHS's current yearly budget - £50 billion – as per Diabetes UK[6]. The Department of Health[7], UK, reports that the cost of overweight and obese individuals to the NHS is estimated to be £4.2 billion annually and this is forecast to more than double by 2050. The British Heart Foundation estimates that of the total 270,000 heart attacks each year caused by the coronary heart disease causes in the UK, 28,000 (10.37%) are directly attributable to obesity[8].

The International Obesity Task Force[9] reports an irony in developing countries: the struggle to reduce hunger in the face of opposing problem of obesity. According to the Diabetes Foundation of India[10] (DFI), 30-50% people in the urban areas are overweight or obese in India. Currently, 38% of the urban population suffer from diabetes and it is expected to rise to 60% by 2025.

The current obesity crisis indicates that there is a pressing need to understand the pathways that control energy homeostasis and to determine the potential to which they can be exploited in the treatment of obesity. Promoting and prolonging satiety is one of the potentially important tactics to tackle obesity. Hence, understanding the physiology behind satiety is imperative in developing treatment strategies for obesity. With the aim of exploring and emphasizing the involvement of oxytocin, a satiety peptide, in appetite regulation (Leng et al., 2008), this study focuses on the effects of some of the appetite regulating peptides on the neurones in the supraoptic nucleus (SON) that synthesize oxytocin.

1.2. Appetite regulating peptides and hypothalamic control

Food is an essential commodity for survival. The storage of energy in the form of adipose tissue is important especially for mammals to maintain constant body temperature. In small mammals, with the exception of hibernators, energy is not adequately retained and thus life is all about procuring and consuming food. Hence, feeding continues as a cyclical process with fall in energy level resulting in initiation of hunger alternating with rise in energy levels and satiety after feeding. The interplay of appetite peptides in signaling the energy status to the brain in these processes is the forerunner of appetite regulation.

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[5] http://www.dh.gov.uk/en/Publichealth/Healthimprovement/Obesity/index.htm
[6] http://www.diabetes.org.uk/en/About_us/News_Landing_Page/National-movement-to-tackle-obesity/
[7] http://www.dh.gov.uk/en/Publichealth/Healthimprovement/Obesity/DH_078098
[8] http://news.bbc.co.uk/1/hi/health/1170787.stm
[9] http://www.iotf.org/documents/iotfsocplan251006.pdf
[10] http://www.diabetesfoundationindia.org/about.htm

The short term meal-related behaviour determines the long term effects such as body weight regulation: short-term control of meal size, meal frequency and the feeling of satiety after a meal are the important determinants of the long-term regulation of body weight. The appetite peptides can be separated into short- and long-term mediators. Short-term mediators are meal related signals that participate in a meal-to-meal control system [e.g. cholecystokinin (CCK)], whereas long-term mediators have a sustained inhibitory effect on food intake and govern body weight and energy metabolism (e.g. leptin). In addition, appetite regulating peptides can be of central [i.e. produced in the hypothalamus/brainstem; e.g. neuropeptide Y (NPY)] or peripheral (i.e. produced in the gut or adipose tissue; e.g. CCK, leptin) origin; they can be orexigenic (e.g. NPY) or anorexigenic (e.g. CCK and leptin).

The signals from the appetite peptides are integrated in the brain, mainly in the hypothalamic nuclei, from where the information is relayed onto higher centres of the brain involved in feeding behaviour. Expression of appetite seems to have been chemically coded in the hypothalamus (Kalra et al., 1999). The hypothalamic nuclei directly involved in appetite regulation are the arcuate nucleus (ARC), lateral hypothalamic area (LHA), dorsomedial nucleus (DMN), ventromedial nucleus (VMN) and paraventricular nucleus (PVN) (Broberger, 2005). The LHA is the hunger centre or feeding centre where as the VMN is the satiety centre (Stellar, 1954; Bray et al., 1990; Flier and Maratos-Flier, 1998). The feeding centre is chronically active and its stimulation can be transiently inhibited after ingestion of food, bringing about satiety (Konturek et al., 2004).

1.3. Oxytocin and vasopressin in homeostasis

Feeding and prandial drinking alter body fluid and electrolyte homeostasis. Hence, intrinsic mechanisms to maintain dynamic equilibrium are essential during feeding to avoid the consequences of altered homeostasis. The best examples for such intrinsic mechanisms for fluid homeostasis are the oxytocin and vasopressin systems (Leng et al., 1988).

The supraoptic nucleus (SON) and the PVN are the two main nuclei where these hormones are synthesized (Fig. 1.1a and 1.1b). The PVN includes smaller parvocellular neurones (pPVN), the axons of which are short and project to other nuclei within the brain or project to the median eminence and secrete releasing hormones [e.g. corticotropin releasing hormone (CRH), thyrotropin releasing hormone (TRF)], and larger magnocellular neurones (mPVN) with longer axons that terminate in the posterior pituitary and secrete oxytocin or vasopressin (Zimmerman et al., 1984). The SON is composed entirely of magnocellular neurones. Nearly all SON and mPVN neurones project to the posterior pituitary. They release either oxytocin or vasopressin into the circulation; however, most of these neurones

express mRNA for both peptides but in very different amounts (Xi et al., 1999). Peripheral oxytocin has been known to be involved in the regulation of reproduction, parturition, milk ejection, fluid homeostasis, pancreatic secretion, cardiovascular control, and stress (Murphy, 1952; Chan and Sawyer, 1962; Nakano and Fisher, 1963; Fendler et al., 1964; Kaneto and Kosaka, 1970; Wakerley et al., 1973). The importance of central oxytocin in the control of reproductive and social behaviours, learning, memory, anxiety and stress was realised more recently (Engelmann et al., 2000; Heinrichs and Domes, 2008; Savaskan et al., 2008) (Fig. 1. 2).

1.4. Central oxytocin and appetite

The importance of oxytocin in appetite regulation as a satiety peptide was recognised from the 1980s when Verbalis *et al* (1986a) found that ingestion of food and CCK administration elevated plasma oxytocin levels. This led them to suggest that satiety might involve activation of hypothalamic oxytocinergic pathways that control the inhibition of ingestion (Verbalis et al., 1986a). Later, interruption of a PVN-hindbrain oxytocinergic projection was found to induce hyperphagia (Kirchgessner et al., 1988). Subsequently, Arletti *et al* (1989) reported that oxytocin influences feeding behaviour in rats (Arletti et al., 1989). Central administration of oxytocin dose-dependently reduced the food intake and the time spent eating, and increased the latency to the first meal in fasted rats (Arletti et al., 1989). Central pretreatment with an oxytocin antagonist prevented the inhibitory effect of central oxytocin on feeding, and increased food intake (Arletti et al., 1989). The inhibition of feeding and drinking after central oxytocin administration was observed in both sexes to about the same degree (Benelli et al., 1991). Later, the anorectic effect of central oxytocin was confirmed by more studies from Olson *et al* (Olson et al., 1991a, b, c) and Verbalis *et al* (Verbalis et al., 1993). Central oxytocin has been found to inhibit salt appetite complementing the peripheral natriuretic actions of oxytocin (Blackburn et al., 1992; Blackburn et al., 1993; Stricker and Verbalis, 1996). Endogenous glucagon like peptide-1 (GLP-1) receptor signaling was suggested to be an important downstream mediator of central oxytocin induced anorexia in rats (Rinaman and Rothe, 2002).

CCK, a peripheral anorectic peptide, was found to activate oxytocin neurones in the SON and PVN (Hamamura et al., 1991). CCK-activated nucleus tractus solitarii (NTS) neurones were shown to send noradrenergic projections to the SON oxytocin neurones (Onaka et al., 1995a) and also to receive oxytocin axon innervation from the PVN oxytocin neurones (Blevins et al., 2003), though these neuronal subpopulations in the NTS may be different. CCK was found to be less effective in inhibiting food intake in the presence of central oxytocin receptor antagonist suggesting the involvement of central oxytocin in CCK-

induced satiety (Blevins et al., 2003). Recently, it was observed by Takashi Higuchi (2009) that bilateral ablation of SON abolished CCK-induced satiety (Takashi Higuchi, *personal communication*). However, CCK-induced reduction in food consumption was not affected in oxytocin-deficient mice (Mantella et al., 2003).

Central administration of an oxytocin receptor antagonist attenuated the effect of leptin on food intake (Blevins et al., 2004). An anorectic dose of leptin administered into the third ventricle increased the number of pPVN oxytocin neurones that expressed Fos, a subset of which projected to the NTS (Blevins et al., 2004). Oxytocin receptor antagonism resulted in a significant decrease in the potentiating effect of leptin on CCK activation of NTS neuronal Fos expression (Blevins et al., 2004). Studies on female oxytocin deficient mice suggested that central oxytocin may be an important inhibitor of sodium consumption (Amico et al., 2003). Genetic absence of oxytocin in male and female mice enhanced their daily consumption of caloric and noncaloric sweet solutions (Amico et al., 2005; Billings et al., 2006; Sclafani et al., 2007). The pattern of consumption suggested that the absence of oxytocin increased sweet and not sweet carbohydrate intake by attenuating or abolishing postingestive satiety-related processes (Sclafani et al., 2007).

SON oxytocin neurones are strongly activated during feeding (Johnstone et al., 2006). Centrally released oxytocin seems to antagonise the actions of ghrelin, a peripheral appetite stimulating hormone, because the orexigenic response to ghrelin was elevated by pretreatment with an oxytocin receptor antagonist (Olszewski et al., 2007). During mid-pregnancy, central oxytocin release is attenuated (Douglas et al., 1995), which may contribute to the hyperphagia that prevails during pregnancy (Douglas et al., 2007), even in the face of increased oxytocin receptor binding and mRNA expression in the hypothalamus (Young et al., 1997; Bealer et al., 2006).

1.5. Source of central oxytocin

Until recently, oxytocin from the pPVN was considered to be the source of brain oxytocin. However, most of the oxytocin in the brain is not in the nerve terminals but in the dendrites of mPVN and SON neurones. The dendrites typically comprise about 80% of the volume of a neurone. SON neurones have 1-3 dendrites projecting to the ventral surface of the brain forming a dense plexus (Armstrong, 1995). At the plexus, the concentration of oxytocin is 100-1000 fold higher than that in blood (i.e. more than 1-10nM). The half life of oxytocin released in the brain is approximately 20min by which time oxytocin effectively spreads to distant brain areas (Mens et al., 1983). Interestingly, axonal and dendritic peptide release of oxytocin can occur independent of each other (Sabatier et al., 2003b; Sabatier and Leng, 2006). While an increase in the electrical activity of oxytocin neurones corresponds to

the peripheral release of oxytocin, dendritic release of oxytocin may or may not accompany an increase in the electrical activity and Fos expression. For example, CCK increases Fos expression and electrical activity of oxytocin neurones accompanied by peripheral (Hamamura et al., 1991) as well as central oxytocin (Neumann et al., 1994) release, but central administration of α-melanocyte stimulating hormone (α-MSH), a central anorectic peptide, inhibits electrical activity and peripheral release while inducing dendritic release of oxytocin and Fos expression (Sabatier et al., 2003b; Sabatier et al., 2003a).

1.6. SON and appetite: peptides/receptors/pathways

Because of the anatomical location of the SON in close proximity with other nuclei involved in appetite regulation (Berthoud, 2002), the concept of central oxytocin release seems even more relevant in the regulation of appetite (Fig. 1.3). Moreover, oxytocin receptors are expressed in the central nervous system (CNS) areas that are involved in appetite regulation, feeding behaviour, learning and memory such as the hypothalamus, brainstem and the limbic regions (Gimpl and Fahrenholz, 2001).

The SON is an outstanding model for the understanding of neuronal function because of its homogeneity, yet it expresses almost every gene that is expressed in the brain (Mutsuga et al., 2004). For example, with regard to appetite regulating peptides, SON neurones are immunoreactive for several of them, including CCK (Vanderhaeghen et al., 1981; Meister et al., 1990), CRH (Lightman and Young, 1987), TRH (Meister et al., 1990), orexin (Backberg et al., 2002) and cocaine amphetamine regulated transcript (CART) (Elias et al., 2001), galanin (Gaymann and Martin, 1989; Meister et al., 1990), dynorphin (Lightman and Young, 1987; Meister et al., 1990), enkephalins (Meister et al., 1990) and neuropeptide FF (Boersma et al., 1993), though the physiological significance of these peptides in the SON is not known. In turn, SON neurones express receptors for several appetite peptides such as leptin (Hakansson et al., 1998), secretin (Chu et al., 2006a, b), CCK (Day et al., 1989), NPY (Wolak et al., 2003), melanocortin-4 receptor (MC4-R) (Mountjoy et al., 1994), orexin (Backberg et al., 2002), CRH (Luo et al., 1994) and PACAP (Nomura et al., 1996). Moreover, the SON receives projections from nuclei that are involved in appetite regulation such as the medial preoptic area (mPOA) (Wilkin et al., 1989; Oldfield et al., 1994), DMN (Honda and Higuchi, 2007), LHA (Stanzani et al., 1984), parabrachial nucleus (Jhamandas et al., 1991) and NTS (Day, 1989; Onaka et al., 1995a; Shioda and Nakai, 1996).

The projections from the NTS, ARC and LHA to the SON and are important because of the significant involvement of these nuclei in appetite regulation:

NTS: The NTS receives direct inputs from the visceral sensory and taste receptors via vagal, glosso-pharyngeal, facial and trigeminal afferents (Contreras et al., 1982). The

vagal afferents are particularly important through which several peripheral appetite peptides such as CCK signal the brain (Monnikes et al., 1997) (Fig. 1.4). The NTS receives strong projections from the area postrema (AP) (Cunningham et al., 1994) that detects peripheral signals via its deficient blood-brain barrier (Faraci et al., 1989). The NTS possesses a significant population of POMC neurones besides the ARC and is one of the nuclei with the highest concentration of MC4-R (Mountjoy et al., 1994). Direct NTS injection of melanocortin receptor agonist reduces and antagonist increases food intake (Williams et al., 2000). The NTS has direct reciprocal connections with other hypothalamic nuclei involved in food intake regulation such as the PVN and LHA (Kannan and Yamashita, 1985; Williams et al., 2001; Buller, 2003). There are no direct projections to the cerebral cortex but there are projections via thalamus, hypothalamus, amygdala and parabrachial cortex (Berthoud, 2002). In addition, the cerebral cortex receives information from the NTS via brainstem arousal systems such as the LC and raphe nucleus (Berthoud, 2002).

ARC: The ARC integrates hormonal signals for energy homeostasis (Cone et al., 2001). The ARC-median eminence (ME) area is one of the circum-ventricular organs (CVO) where the blood-brain barrier is deficient, allowing entry of blood-borne chemicals and hormones such as leptin (Schwartz et al., 1992; Friedman and Halaas, 1998). The ARC contains mainly two populations of neurones: NPY-agouti related peptide (AgRP) and POMC-CART neurones. AgRP is the endogenous antagonist for MC4-R (Rossi et al., 1998). NPY-AgRP neurones promote food intake and inhibit the anorexigenic effect of α-melanocortin stimulating hormone (α-MSH) from POMC neurones via MC4-R antagonism by AgRP. A subpopulation of ARC-NPY neurones expresses leptin receptors (Ob-Rb) (Mercer et al., 1996). Leptin reduces NPY mRNA (Wang et al., 1997) and increases POMC mRNA level (Schwartz et al., 1997; Mizuno et al., 1998) in ARC neurones. One of the derivatives from POMC is α-MSH (Bjorbaek and Hollenberg, 2002), in response to which the dendrites of SON neurones release oxytocin (Sabatier et al., 2003b).

LHA: The LHA contains two distinct neuronal populations containing either orexins (orexin A and B) or melanin concentrating hormone (MCH) both of which stimulate food intake upon central administration (Qu et al., 1996; Dube et al., 1999) and their mRNA levels are increased by fasting (Herve and Fellmann, 1997; Cai et al., 1999). Orexins are involved in the regulation of arousal as well (Kohlmeier et al., 2004).

VMN: The neuronal populations of the VMN are affected by many appetite peptides such as leptin, ghrelin, orexin-A and CCK (King, 2006). If feeding accompanies central oxytocin release, the primary target could be the VMN (Sabatier et al., 2007), the satiety centre, because the VMN is one of the most prominent sites of oxytocin receptor expression

(Bale et al., 1995a, b). α-MSH-induced central oxytocin from the SON diffuses to the VMN (Nancy Sabatier, *Personal communication*) and the electrical activity of VMN neurones is altered in response to central oxytocin (Sabatier et al., 2007). It is not known whether VMN neurones project to the SON.

The finding that the SON receives direct and/or indirect projections from these centres that are directly involved in appetite control signifies the potential importance of the SON in appetite control too.

1.7. Oxytocin, satiety and reward

The termination of a meal and the feeling of satiety do not depend on the nutritive or calorific value of the food (Anderson and Woodend, 2003). It seems that it is the presence of food and associated cascade of taste, stretch and hormonal signals following the food intake that mediate satiety. Lacking the sense of contentment after food intake or satiety could be one of the important causes of obesity (Moran et al., 1998). Satiety associated with food intake is a natural reward[11] (Kelley and Berridge, 2002) that is essential for sustaining procurement processes, which otherwise will be terminated without a reward. Oxytocin is one peptide that is implicated in satiety, feeding behaviour and reward (Marazziti and Catena Dell'osso, 2008). In addition, SON oxytocin neurones are activated by oropharyngeal stimulation (Naimi et al., 1997), gastric distension and peripheral post-prandial signals such as CCK (Renaud et al., 1987), all of which contribute to satiety.

1.8. Oxytocin lacks attention as an anorectic peptide

Despite the accumulating evidence for the involvement of oxytocin neurones in regulation of food intake, the primary hypothalamic sources of oxytocin, such as the mPVN and SON, are not generally considered as important centres involved in appetite regulation (Kalra et al., 1999; Schwartz, 2001; Berthoud, 2002; Grill and Kaplan, 2002; Saper et al., 2002; Appleyard, 2003; Luckman and Lawrence, 2003; Jobst et al., 2004; Leibowitz and Wortley, 2004; Williams et al., 2004; Arora and Anubhuti, 2006; Morton et al., 2006), except for passing reference to a role of the mPVN in appetite regulation (Kalra et al., 1999; Berthoud, 2002). Furthermore, except for the recent reviews by Douglas *et al* and Leng *et al* (Douglas et al., 2007; Leng et al., 2008), oxytocin is not given much attention as an anorectic signal (Kalra et al., 1999; Berthoud, 2002; Grill and Kaplan, 2002; Saper et al., 2002; Appleyard, 2003; Leibowitz and Wortley, 2004; Williams et al., 2004; Arora and Anubhuti, 2006; Morton et al., 2006).

[11] Definition of a reward: the willingness to engage in otherwise unrelated behaviours that quickly extinguish in the absence of the reward [Saper CB, Chou TC, Elmquist JK (2002) The need to feed: homeostatic and hedonic control of eating. Neuron 36:199-211.].

Recently, the presence of oxytocin and oxytocin receptors have been established in the enteric nervous system (Welch et al., 2009) the functional significance of which is yet to be explored. It has been well established that oxytocin neurones are activated in response to gut peptides such as CCK, secreted after food intake (Hamamura et al., 1991). However, oxytocin is not generally considered as a neurohormone integral to the brain-gut axis (Havel, 2001; Konturek et al., 2004; Arora and Anubhuti, 2006; Naslund and Hellstrom, 2007; Wren and Bloom, 2007).

1.9. Role of peripheral oxytocin and vasopressin in osmoregulation

Osmotic stimuli activate SON and PVN oxytocin neurones and increases peripheral release of oxytocin (Brimble et al., 1978; Verbalis et al., 1986a; Russell et al., 1988). Oxytocin administration results in a dose-dependent increase in urine flow (diuresis) and Na^+ and Cl^- excretion (natriuresis) in conscious and anaesthetized rats (Balment et al., 1980; Conrad et al., 1986; Verbalis et al., 1991; Windle and Forsling, 1991; Forsling et al., 1994; Huang et al., 1995; Windle et al., 1997). SON neurones in the rats are osmosensitive (Mason, 1980) and osmoreceptor activation is involved in the stimulation of oxytocin and vasopressin release following osmotic stimulation (Negoro et al., 1988; Bourque et al., 1994).

Oxytocin mediates its natriuretic and diuretic effects either by direct or by indirect means: 1. Oxytocin acts on its receptors in the nitric oxide (NO)-ergic cells of macula densa and proximal tubules and activates renal NO synthase leading to generation of NO that increases cGMP (at higher doses, oxytocin increases the release of ANP that in turn also increases cGMP). cGMP mediates decreased tubular Na+ reabsorption by closing Na+ channels and thus results in natriuresis (Soares et al., 1999). Regulation of renal kallikrien-kinin system by oxytocin could be another direct mode of action. Urinary kallikrien and kinin levels are also increased during oxytocin-induced diuresis. Diuretic and natriuretic actions of oxytocin were significantly attenuated by administration of kallikrien inhibitor (Adachi et al., 1995). 2. Indirectly through ANP: Blood volume expansion and baroreceptor activation causes the release of ANP from ANPergic neurones in the hypothalamus that in turn stimulates release of oxytocin from the posterior pituitary. Oxytocin acts on the heart to release ANP from the right atrium. ANP has negative chronotropic and ionotropic effects on the heart and thus reduces cardiac output, thereby reducing effective circulating blood volume and also it promotes natriuresis in the kidneys (Haanwinckel et al., 1995; Antunes-Rodrigues et al., 1997; Favaretto et al., 1997; McCann et al., 1997).

Vasopressin is antidiuretic but synergistic with oxytocin in natriuresis (Gross and Anderson, 1982; Balment et al., 1984; Balment et al., 1986; Brimble et al., 1988; Windle et al., 1995). Vasopressin neurones are inhibited when peripheral baroreceptors are activated

(Jhamandas and Renaud, 1986, 1987) and vasopressin release is stimulated during haemorrhage to preserve effective blood volume through its antidiuretic and hypertensive effects (Fyhrquist et al., 1981).

As the fluid homeostasis is altered during and after food intake and oxytocin is released postprandially to regulate this alteration, the changes in the electrical activity of oxytocin and vasopressin neurones in response to appetite peptides will reflect the physiological fluid homeostatic changes taking place around feeding.

1.10. Advantages of *in vivo* electrophysiology

The technique of *in vivo* electrophysiology was chosen because of its technical and physiological advantages such as the ability in (1) showing the real-time activity of neurones, (2) showing clear cut excitation or inhibition unlike Fos expression which is not confirmatory of either excitation or inhibition, (3) showing the duration of excitation or inhibition of neurones in real-time upon administration of a drug, unlike Fos expression which begins long after initiation of an activation signal and in (4) providing single cell resolution unlike other real-time imaging studies which lack resolution, (5) permitting all the synaptic inputs and contacts to stay intact (apart from anaesthetic effects) *in vivo* unlike *in vitro* electrophysiological studies where the system lacks synaptic inputs (6) reflecting peripheral release of the peptide and finally (7) the preparation allows physiological (systemic route) methods of administration of drugs unlike *in vitro* studies where only direct application is possible which may not be physiological.

1.11. Summary

Though independent studies indicate that oxytocin does play a significant role in appetite regulation and fluid homeostasis during and after food intake, recent reviews in the literature ignore the role of oxytocin in energy and water homeostasis. Hence, more studies with a focus on SON oxytocin neurones are required to emphasize the relationship between oxytocin and appetite control. Therefore, this study was planned to explore the responses of SON neurones to four different appetite peptides of central and peripheral origin involved in stimulating or inhibiting appetite or in the signalling between the gut and the brain (Fig. 1.5):

1. Neuropeptide Y - central orexigenic peptide.
2. Leptin - peripheral anorexigenic peptide from adipose tissue.
3. Secretin - a brain-gut peptide.
4. CCK - peripheral anorexigenic peptide from the gut.

Detailed background information is given in the introductions to the respective chapters, however, the main objectives are stated below. Although the focus is on the SON oxytocin neurones, the effects of appetite peptides on SON vasopressin neurones were also

recorded and reported. Also, changes in oxytocin neurone responsiveness were investigated in response to some of the appetite peptides in states of extreme differences in food intake, such as late pregnancy and fasting.

Neuropeptide Y: Central administration of NPY, the most potent orexigenic peptide, was found to elevate plasma oxytocin levels (Parker and Crowley, 1993). However, the plasma oxytocin response to central NPY is attenuated during pregnancy (Brunton et al., 2006a), similar to the attenuation seen in the responses to various other stimuli (Russell et al., 2008). To study whether NPY alters the electrical activity of SON oxytocin neurones and to study whether the increase in electrical activity is attenuated during late pregnancy, responses of SON oxytocin neurone to NPY were studied in virgin and late pregnant rats.

Leptin: The effect of systemic leptin on the electrical activity of SON oxytocin neurones is not known. As pregnancy is an interesting model to study the effects of leptin [i.e. a model of hyperleptinemia together with leptin resistance, a model of physiological hyperphagia despite high leptin levels (Ladyman and Grattan, 2004), a model of attenuated oxytocin response to various stimuli (Russell et al., 2008) and a model of altered fluid homeostasis (Brunton et al., 2008)], the effects of systemic administration of leptin on SON oxytocin neurones was also studied in late pregnant rats. As it is reasonable to assume that reduction in circulating leptin levels by fasting (Ladyman and Grattan, 2004) would increase the sensitivity to exogenous leptin administration, the effects of leptin were studied in fasted virgin and late pregnant animals as well.

Secretin: Recently, secretin, a brain-gut peptide, has been found to induce Fos expression in the SON (Goulet et al., 2003). Central administration of secretin *in vivo* elevated plasma oxytocin level and direct application of secretin increased oxytocin release from hypothalamic explants *in vitro* (Takayanagi and Onaka, 2007). However, the doses used in these studies were supraphysiological (40µg systemically or up to 1-10µg centrally). Hence, the effects of more physiological doses of secretin (less than 1µg; i.v) on the electrical activity of SON neurones were investigated. The secretin induced effects on SON neurones were expected to be similar to CCK induced effects as these peptides are physiologically similar. In addition, the neurochemical pathway involved in the secretin mediated SON neuronal responses was explored. The plasma oxytocin changes upon systemic administration of secretin were also studied in urethane-anaesthetized virgin rats to correlate with the electrical activity of SON oxytocin neurones.

Interactions: As leptin and CCK are synergistic in reducing food intake and body weight (Barrachina et al., 1997; Matson et al., 1997; Matson and Ritter, 1999; Matson et al., 2002), interaction between leptin and CCK on the CCK-induced excitation of SON oxytocin

neurones was sought. Also, the effect of reduction in the endogenous level of leptin by fasting on the excitation of SON oxytocin neurones induced by CCK and secretin was also studied. In addition, as fasting is a stressor and oxytocin is a stress hormone (Lang et al., 1983), the basal activity of oxytocin neurones would be expected to be higher in the fasted rat model. On the other hand, as fasting also reduces fluid intake (Apostolou et al., 1976; Maejima and Nagase, 1991; Burlet et al., 1992; Youn and Buchanan, 1993) basal activity of oxytocin neurones would be expected to be lower to avoid natriuretic effects of oxytocin. Hence, the effect of fasting on the basal and peptide-induced change in activity of oxytocin neurones was also observed. The SON oxytocin responses observed in fasted/unfasted rats upon administration of leptin and secretin were compared with the CCK-induced excitatory responses.

1.12. Hypotheses

1.12.1. Neuropeptide Y

1. Central administration of NPY increases the electrical activity of SON oxytocin neurones in urethane-anaesthetized virgin rats and the response is attenuated in late-pregnant rats.
2. Systemic administration of NPY increases the electrical activity of SON oxytocin neurones in urethane-anaesthetized virgin rats *in vivo*.
3. Central administration of NPY increases while systemic administration of NPY decreases the electrical activity of SON vasopressin neurones in urethane-anaesthetized virgin rats *in vivo*.

1.12.2. Leptin

1. Systemic leptin, a long-term satiety signal, does not affect the electrical activity of SON oxytocin and vasopressin neurones in virgin rats.
2. SON oxytocin neurones are resistant to the effects of systemic leptin during late pregnancy.
3. Leptin-induced effects in the SON oxytocin neurones are either positively (due to increased sensitivity) or negatively (to prevent natriuresis) altered by fasting in virgin and late-pregnant rats.
4. Leptin-induced changes in the electrical activity of SON oxytocin neurones in fasted rats are dose dependent.

1.12.3. Secretin

1. Systemically administered secretin, at physiological doses, increases the electrical activity of SON oxytocin neurones and either inhibits or does not affect vasopressin neurones, similar to CCK.

2. Secretin dose-dependently excites SON oxytocin neurones and increases peripheral release of oxytocin in urethane anaesthetized female rats.
3. Systemic secretin does not induce somato-dendritic oxytocin release from the SON.
4. Similar to CCK, central noradrenergic pathways are involved in the secretin-induced excitation of SON oxytocin neurones.
5. Basal activity and secretin-induced excitation of SON oxytocin neurones are attenuated in fasted state.

1.12.4. Cholecystokinin

1. CCK-induced excitation of SON oxytocin neurones are altered during fasting in virgin and pregnant rats.
2. Systemic leptin potentiates the CCK-induced excitatory responses of SON oxytocin neurones.

1.13. Objectives

1.13.1. Neuropeptide Y (NPY):

1. To study the effect of central administration of NPY on the electrical activity of SON oxytocin neurones in urethane-anaesthetized virgin and late-pregnant rats *in vivo*.
2. To study the effect of systemic administration of NPY on the electrical activity of SON oxytocin neurones in urethane-anaesthetized virgin rats *in vivo*.
3. To study the effect of central and systemic administration of NPY on the electrical activity of SON vasopressin neurones in urethane-anaesthetized virgin rats *in vivo*.

1.13.2. Leptin

1. To study the electrophysiological responses of SON oxytocin neurones to systemic administration of leptin *in vivo* in unfasted/fasted virgin/pregnant rats.
2. To study whether leptin-induced changes in electrical activity of SON oxytocin neurones in fasted rats are dose dependent.
3. To study the electrophysiological responses of SON vasopressin neurones to systemic administration of leptin *in vivo*.

1.13.3. Secretin

1. To study the electrophysiological responses of SON oxytocin and vasopressin neurones to intravenous administration of secretin in urethane anaesthetized female rats *in vivo.*
2. To evaluate whether secretin-induced effects on SON oxytocin neurones and plasma oxytocin concentration in urethane-anaesthetized female rats are dose dependent.

3. To study the systemic secretin-induced somato-dendritic oxytocin release from the SON.
4. To study the involvement of adrenergic pathways in secretin-induced effects on SON oxytocin neurones in urethane anaesthetized female rats.
5. To study the basal activity and secretin-induced change in the activity of SON oxytocin neurones in fasted rats.

1.13.4. Cholecystokinin

1. To study the electrophysiological responses of SON oxytocin neurones *in vivo* after systemic administration of CCK in fasted urethane-anaesthetized virgin and pregnant rats and to compare with unfasted virgin and pregnant rats.
2. To study whether systemic administration of leptin potentiates the CCK-induced excitatory electrophysiological responses of SON oxytocin neurones in unfasted virgin rats.
3. To compare the electrophysiological responses of SON oxytocin and vasopressin neurones in unfasted/fasted virgin rats to systemic administration of secretin and leptin with the responses to CCK administration.

CHAPTER II

METHODS

2.1. Animals

Adult (≥10 weeks old) female Sprague-Dawley rats of 200-300g body weight were obtained from the colony maintained at Hugh Robson Building – Biomedical Research Resources, University of Edinburgh, UK. The rats, six per cage, were housed under 12h light and 12h dark cycle (lights on at 7 AM). Temperature was maintained at 19-23^{o}C and the humidity at 55 ± 10%. The rats had free access to feed and water except when they were fasted when they had access only to water.

Random cycling female rats were used for the experiments as routine vaginal cytology was not feasible. The influence of altered steroidal status during different phases of cycle on the electrophysiological response of oxytocin neurones to the peptides studied was considered negligible as the responses in virgin rats have been observed to be consistent and homogeneous.

The females were introduced into a male cage for mating. The day a vaginal plug found was noted as day 0 with the expected day of parturition being day 22. Late pregnant rats of day 19-21 were used for experiments. All procedures were performed in accordance with current UK Home Office regulations under the Animals (Scientific Procedures) Act 1986 and local ethical committee approval.

2.2. Drugs

2.2.1. Neuropeptide Y (NPY; Porcine)

NPY was obtained from Tocris Bioscience (Bristol, UK) as a freeze-dried aliquot containing 200µg of NPY. This was resuspended in distilled water and stored at -20^{o}C in 20µg/4µl aliquots. For intracerebroventricular (i.c.v; lateral cerebral ventricle) use, 4 µl of 2X aCSF (See Appendix) was added and NPY was administered at a dose of 5µg/2µl. NPY was used at a dose of 20µg/rat in 100µl saline for i.v. administration.

2.2.2. Recombinant Rat Leptin

Leptin was obtained from Peprotech (France) as a freeze-dried aliquot containing 1mg of leptin. This was resuspended in normal saline (0.9% w/vol) and stored at 4^{o}C. Leptin was administered i.v. at 1µg, 10µg, 100µg or 1mg per rat in 100µl saline.

2.2.3. Secretin (Rat)

Secretin was obtained from Tocris Bioscience (Bristol, UK) as a freeze-dried aliquot containing 1mg of secretin. This was resuspended in distilled water and stored at -20^{o}C in 1µg/10µl aliquots. Secretin was administered i.v. at 0.01µg, 0.1µg or 1µg per rat in 100µl saline.

2.2.4. Cholecystokinin (CCK) Octapeptide (Sulfated)

CCK was obtained from Tocris Bioscience (Bristol, UK) as a freeze-dried aliquot containing 1mg of CCK. This was resuspended in distilled water and stored at -20^{o}C as 25µg/50µl aliquots. For use, 450µl saline was added to an aliquot. CCK was administered i.v. at a dose rate of 25µg/kg.

2.2.5. Benoxathian hydrochloride

Benoxathian was obtained from Sigma (Dorset, UK) as a freeze-dried aliquot containing 25mg of benoxathian. This was resuspended in distilled water and stored at -20^{o}C as 2µmoles/50µl (800µg/50µl) aliquots. For microdialysis use, 950µl aCSF was added to make a 2mM solution. For i.c.v. use, 50µl of 2X aCSF was added and administered at a dose rate of 8µg/µl min^{-1}.

2.3. In-vivo electrophysiology

2.3.1. Surgical procedure

Anaesthesia was induced by exposure to halothane (Halothane-VET, Merial Animal Health Ltd, Essex, UK) vapour in an anaesthetic chamber. The rats were then anaesthetized by intraperitoneal (i.p) injection of urethane (ethyl carbamate; 25% w/v; 1.25g/kg; Sigma). The left femoral vein was cannulated for intravenous drug administration and the cannula attached to a syringe (1ml) containing normal saline (0.15M; 0.9% NaCl). An endotracheal tube was inserted into the trachea to keep the airway patent.

Using the ventral surgical approach (Leng and Dyball, 1991), the ventral surface of the SON and neural stalk were exposed (Fig. 2.1). The anaesthetised rat was fixed in a stereotaxic frame in the supine position. The ramii of the mandible were separated and the tongue was pulled between the lower incisors and held using a retractor. The lower incisors were parted and secured laterally with wires. Using thermal cautery, the soft palate and tissues covering the hard palate were cauterized. A dental drill was used to remove a 3mm diameter area of the hard palate. The lateral wing of the palatine bone covering the trigeminal nerve was also removed. The trigeminal nerve was severed to expose the ophthalmic vein which was blocked at both ends using bone wax and then severed. This exposed the meningeal covering of the ventral surface of the brain. The optic chiasma, seen as a white band, and a cerebral artery were visually discernible through the meninges. The SON is located lateral to the suture between the presphenoid and basi-sphenoid bones and it lies along the lateral border of the optic chiasma beneath the cerebral artery. A slit was made using a bent 26G needle close to the optic chiasma and adjacent to the cerebral artery to insert a recording electrode.

A shallow hole was drilled in the basi-sphenoid bone posterior to the suture between the presphenoid and basisphenoid bones. This required blocking the venous sinus that traverses the bone with bone wax. The median eminence, neural stalk and a part of the posterior pituitary were exposed once the thin bony sheet was removed.

2.3.2. In-vivo electrophysiological recording

SON neurones were antidromically driven by giving constant current biphasic rectangular pulses (1mA for duration of 1ms) via a tungsten bipolar stimulating electrode (Series 200, Model SNEX200; Pfeffer Science Products - Gmbh, Hofheim, Germany) placed in the neural stalk. A glass capillary (GC150F-10; 1.5mm outer diameter; 0.86mm inner diameter; Harvard Apparatus Limited, Kent, UK), pulled on a magnetic puller (Narishige Scientific Instrument Laboratory, Tokyo, Japan) into a recording microelectrode with a tip diameter of approximately 1µm and filled with 0.15M NaCl, was lowered into the SON. A Digitimer D130 spike processor was used to detect spikes. Signals were amplified and then filtered using a 50Hz noise eliminator (Hum Bug; Quest Scientific, Canada). The signal was passed through an interface device (CED1401; Cambridge Electronic Design, Cambridge, UK) to a computer. For a single rat, a minimum of one to a maximum of three neurones were recorded. Hence, the 'n' number indicates the number of neurones recorded and not necessarily, but normally, the number of rats. The rats were killed by anaesthetic overdose (Pentobarbitone; 60mg/kg; i.v; Euthatal, Merial Animal Health Ltd, Essex, UK) at the end of the experiments.

2.3.3. Identification of SON neurones

SON neurones were identified by antidromic stimulation of the neural stalk with 1mA current for duration of 1ms every 3s (Fig. 2.2). Each stimulation of the neural stalk evokes an action potential in the axon, which is propagated to the cell body (Yagi et al., 1966). An antidromic spike occurring at a constant latency confirmed the cell as a SON neurone. All SON neurones displayed positive-going spikes of prolonged and complex action potentials and most of them showed a distinct notch on the descending slope representing a prominent calcium component (Mason and Leng, 1984; Leng and Dyball, 1991).

2.3.4. Differentiation of SON oxytocin neurones from vasopressin neurones

SON oxytocin neurones were differentiated from vasopressin neurones by their non-phasic firing pattern, shape of the inter-spike interval histogram and hazard and by an excitatory response to i.v. cholecystokinin (CCK octapeptide, sulphated; Tocris; 25µg/kg).

2.3.4.1. Firing pattern

The typical firing pattern of vasopressin cells is known as 'phasic firing' (Fig. 2.4). Bursts of firing are separated by silent phases in between (Leng and Dyball, 1991). Oxytocin neurones fire in a continuous manner i.e. in a non-phasic pattern (Fig. 2.3). But some vasopressin neurones are also continuously active and these can be differentiated from the oxytocin neurones using other parameters described below.

2.3.4.2. Interspike interval histogram

For oxytocin neurones, the descending tails of the interspike interval distributions can be fitted well using a single negative exponential (Leng et al., 2001) (Fig. 2.5a – 2.6b). This shape of interval histogram suggests that spikes are generated randomly but subject to post-spike hyperpolarization (Sabatier et al., 2004).

For vasopressin neurones, the tails of the interval distributions cannot be fitted well using a single exponential (Leng et al., 2001) (Fig. 2.7a – 2.10b). The interspike interval histogram is highly skewed. This shows that the spike patterning is dominated by a sequence of post-spike refractoriness followed by hyperexcitability, consistent with a sequential hyperpolarization and depolarization (Sabatier et al., 2004).

2.3.4.3. Hazard analysis

Hazard is the probability of occurrence of a spike immediately after a spike that has already occurred. Hazard functions use the same data as interval histograms, but, in each bin, plot the incidence of spikes as a proportion of the size of the subsequent tail of the histogram (Sabatier et al., 2004). A hazard function is calculated using the formula:

$$h_{[i-1,\,i]} = n_{[i-1,\,1]}/(N - n_{[0,\,i-1]})$$

where $h_{[i-1,\,i]}$ is the hazard at interval i, $n_{[i-1,\,1]}$ is the number of spikes in interval i, $n_{[0,\,i-1]}$ is the total number of spikes preceding the current interval and N is the total number of spikes in all intervals (Brown et al., 2008). This gives the inferred probability (as a decimal) of a cell firing a subsequent spike in any interval after a spike (at time 0), given that another spike has not occurred earlier (Brown et al., 2008).

A constant hazard is produced by a completely random firing pattern. A positive shift in the hazard function (i.e. an increased hazard at any given time after the preceding spike without a change in the shape of the hazard plot) infers a step increase in the probability of a spike firing (i.e. of the neuron reaching threshold), which might result from increased (random) synaptic drive and/or a sustained depolarization upon which continued/increased (random) synaptic drive is superimposed. Divergence above or below a constant hazard reveals periods of increased and decreased post-spike excitability,

respectively, which might result from changes in intrinsic membrane properties following a spike (Brown et al., 2008).

Ratios of peak early (<0.07s inter-spike interval) to mean late (0.2–0.3s inter-spike intervals) hazards (Brown et al 2008) were calculated before and after drug administration for each neuron to seek changes in the shape of the hazard plot.

Hazard functions for oxytocin neurones show an initial refractory period, then a steady increase in hazard to a plateau after a post-spike interval of about 50ms, implying that after about 50ms, there are no detectable excitatory effects of an individual spike upon neuronal excitability (Sabatier et al., 2004) (Fig. 2.11a). The seemingly increasing average hazard constructed for oxytocin neurones in the present study (Fig. 2.11b) is an artefact of truncation of interspike interval data to 0.5s. Hazard analysis of vasopressin neurones show a low probability of discharge immediately after a spike, indicating a refractory period, followed by a peak of increased probability after each spike (from about 10 to 50 ms), which then decreases (Fig. 2.12a).

The different hazard plots exhibited by SON oxytocin (Fig. 2.11a and b) and vasopressin neurones were used to aid identification of the neurones (Fig. 2.12a, b, c and d).

2.3.4.4. Response to intravenous CCK

Oxytocin neurones are transiently excited (Fig. 2.13) and vasopressin neurones are transiently inhibited (Fig. 2.14), or not affected by intravenous administration of cholecystokinin (CCK; Tocris; 25µg/kg) (Renaud et al., 1987). This test was used to distinguish oxytocin and vasopressin neurones.

2.3.5. Analysis of firing rate

2.3.5.1. Oxytocin and non-phasic firing vasopressin cells

Firing rates were recorded and analysed using Spike 2 software (Cambridge Electronic Design) and averaged in 30 second bins. The mean change in firing rate (spikes per second) was calculated for the basal activity (over 10 min) and the periods following each injection.

2.3.5.2. Phasic firing vasopressin cells

Firing rates were recorded and analysed using the W-BURSTS script of the Spike 2 software (Cambridge Electronic Design). The settings were adjusted appropriately to the duration of phasic firing of a particular neurone. The settings defined for most of the analyses were as follows: Maximum interval to identify start of the burst: 1s; Maximum interval between spikes in any one burst: 1s; Minimum interval between the bursts: 5s; Minimum burst duration: 5s; Minimum number of spikes per burst: 20. The number of bursts found, the percentage of spikes in bursts, mean number of spikes per burst, mean burst

length (ms), mean interburst interval (ms) and mean cycle time (ms) were calculated using this analytical software.

Three criteria were employed to evaluate changes in the firing of a phasic firing vasopressin neurone before and after administration of a drug:

1. Activity Quotient (AQ): The ratio of active period to total period.

Activity Quotient = Mean burst length (s)/ Total period of observation (s)

2. Frequency within bursts (spikes/s): Mean intraburst frequency of spikes.

Frequency within bursts (spikes/s) = Mean number of spikes per burst/ Mean burst length (s)

3. Mean interburst interval (s): Average period of silence between the bursts.

2.4. Microdialysis

As for the *in vivo* electrophysiology recording, rats were anaesthetized and the ventral surgical procedure was carried out. An in-house designed U-shaped microdialysis probe was prepared (total membrane length 2.0mm; inner diameter 200µm; mean pore diameter: 4-6nm; Cuprophan® RC55, Membrana, GmBH, Germany). The loop of the probe was positioned on the ventral surface of SON after opening the meninges. In the experiments combining *in vivo* electrophysiology and microdialysis, the recording electrode was positioned within the loop while recording from SON neurones (Fig. 2.15). The SON was dialysed with artificial cerebrospinal fluid (aCSF) or benoxathian hydrochloride (2mM; Sigma, Dorset, UK) dissolved in aCSF, at a flow rate of 3µl/min using a mini-pump. For experiments involving collection of microdialysate, 90µl samples were collected once every 30min. The rats were killed by anaesthetic overdose (Pentobarbitone; 60 mg/kg; i.v; Euthatal, Merial Animal Health Ltd, Essex, UK) at the end of the experiments.

2.5. Intracerebroventricular (i.c.v) cannulation

A urethane-anaesthetized (1.25g/kg; i.p) rat was positioned in a stereotaxic frame in the prone position. A midline skin incision was made on the skull from the level of ears to the forehead exposing bregma and lambda. A guide cannula (22G with a ventral projection of 3.5mm; Bilaney Consultants Ltd, Kent, UK) was positioned 1.6mm lateral and 0.6mm caudal to the bregma and held in place by dental cement supported by two stainless screws inserted into the skull. A dummy cannula (Bilaney Consultants Ltd, Kent, UK) was inserted to keep the guide cannula closed until use. A 28G internal cannula (Bilaney Consultants Ltd, Kent, UK) which projected 1mm below the tip of the guide cannula was used for i.c.v. administration of vehicle or a drug. At the end of the experiment, in some rats, dye was injected to make sure that the tip of the cannula was in the correct location, i.e. in the cerebral ventricle.

2.6. Blood sampling (with Dr. Paula J Brunton)

Rats were anaesthetised with urethane (25%; 1.25g/kg; i.p). The left femoral vein was cannulated with Silastic cannula (0.5 mm internal diameter; 0.25 mm wall) connected to a syringe filled with heparinised (Heparin Injection B.P., Leo Laboratories, Dublin; 50U/ml) 0.9% sterile saline. A minimum of 2h of stabilization period was allowed before taking any sample. 0.3ml of blood was collected for each sample in a syringe containing 0.03ml heparinised saline. Blood was centrifuged immediately; plasma was separated and stored at -20^{o}C until assayed. At the end of the experiments, rats were killed by anaesthetic overdose (Pentobarbitone; 60mg/kg; i.v; Euthatal, Merial Animal Health Ltd, Essex, UK).

2.7. Oxytocin radioimmunoassay (RIA): for plasma oxytocin (with Dr. Paula J Brunton)

This method was adapted from that described by Higuchi *et al* (Higuchi et al., 1985). The first rabbit antioxytocin antibody THF-3, kindly supplied by Prof. Takashi Higuchi (University of Fukui, Japan), diluted to a final concentration of 1: 200,000 in RIA buffer, was added to tubes containing standard oxytocin (National Institute for Biological Standards and Control, Hertfordshire, UK; range 2.4–2500pg/ml; in triplicate) or test samples (in duplicate). After 24h incubation at 4^{o}C, radioiodinated oxytocin (^{125}I, 3.7KBq/ml, 50µl; Perkin Elmer, Life and Analytical Sciences, Buckinghamshire, UK) was added to all of the tubes. After 48h incubation at 4^{o}C, a second donkey anti-rabbit antibody (IDS Ltd, Boldon, UK) at a dilution of 1:25 in assay buffer was added and tubes were kept at 4^{o}C for 24h incubation. At the end of that incubation period, the antigen-antibody complex was precipitated by adding standardized Pansorbin cells (Calbiochem Ltd, Nottingham, UK) diluted to 1:25 in assay buffer. Radioactivity of the precipitate was measured with a γ-counter (Wizard^{TM} 1470 Automatic Gamma Counter, Perkin Elmer) and unknown oxytocin concentrations were read directly from the standard curve constructed automatically by the Ultraterm 2 software package (Wallac Oy, Turku, Finland) (Fig. 2.16). The sensitivity of the assay was 2.4pg/ml and the intraassay variation was less than 14%. All samples from an experiment were assayed in a single assay to avoid interassay variation. [Refer to Appendix for details on reagents]

2.8. Oxytocin radioimmunoassay (RIA): for somato-dendritic oxytocin release from the SON (Done at Prof. Rainer Landgraf's Lab, Germany)

The microdialysate (90µl/tube) samples were sent in dry ice (-79 ^{o}C) to Prof. Rainer Landgraf's Lab (Max Planck Institute of Psychiatry, Germany) for oxytocin RIA. The RIA was done as per the procedure in Landgraf *et al* (Landgraf et al., 1995). The assay sensitivity

was 0.1pg/tube. The intra-assay coefficient of variation was 5-8% and inter-assay coefficient of variation was 8-12%.

2.9. Statistical analysis

Data are represented as mean ± standard error of the mean (s.e.m). Statistical tests were performed on the SigmaStat® software version 3.10 (Systat Software Inc., London, UK). While comparing, the average responses from individual cells of a group were compared with that of another group. Statistical data were analyzed by paired t-test, one-way or two-way ANOVA and one-way repeated measures (RM) ANOVA where appropriate. The significance level for all statistics was set at $P<0.05$.

CHAPTER III

NEUROPEPTIDE Y

3.1. Introduction

Neuropeptide Y (NPY) (Tatemoto, 1982; Tatemoto et al., 1982) is a ubiquitous hormone that has both central and peripheral effects (Kuo et al., 2007). It is a 36-amino acid peptide belonging to the pancreatic polypeptide family (Tatemoto, 1982). It is the most potent appetite stimulant known (Kalra et al., 1991; Flynn et al., 1999). NPY is one of the most conserved peptides during evolution (Larhammar, 1996) suggesting that it is important for the regulation of essential physiological functions. It reacts with a family of G-protein-coupled receptors (GPCR) named from Y_1 to Y_6 (Michel, 1991).

Normally, the orexigenic peptides such as NPY, orexin and ghrelin activate the hypothalamo-pituitary-adrenal (HPA) axis (Haas and George, 1987, 1989; Suda et al., 1993; Johnstone et al., 2005; Spinazzi et al., 2006). The resulting glucocorticoid secretion enhances glucose availability (Munck and Koritz, 1962) ensuring energy homeostasis during periods of reduced availability of food. However, during late pregnancy, the HPA and oxytocin responses to central administration of the orexigenic peptides orexin A (Brunton and Russell, 2003), NPY (Brunton et al., 2006a) and ghrelin (Bales et al., 2006a) are suppressed. The HPA and oxytocin secretory responses to NPY and orexin are absent in late pregnant rats, although the ingestive behavioural responses remain unaltered (Brunton and Russell, 2003; Brunton et al., 2006a). The oxytocin secretory responses to central administration of NPY are reduced during late pregnancy, which may contribute to minimizing the risk of preterm labour and preserve the accumulated neurohypophysial stores of oxytocin for requirement during parturition (Brunton et al., 2006a).

The electrophysiological responses of oxytocin neurones to physically stressful stimuli such as intravenous injection of IL-1β are also attenuated during late pregnancy in urethane-anaesthetized rats (Brunton et al., 2006b). A tonic endogenous μ-opioid inhibition of oxytocin neurone activity emerges centrally towards the end of pregnancy and is exerted at the presynaptic opioid receptors on the NA-ergic nerve terminals in the SON (Brown et al., 2000a). This opioid tone attenuates the IL-1β-induced OT neuronal (secretory and electrophysiological) responses during pregnancy, and administration of naloxone reverses this attenuation (Brunton et al., 2006b). It is not known whether central NPY influences the electrical activity of SON oxytocin neurones and whether NPY-induced responses are attenuated during pregnancy.

Injection of NPY into the SON increases the firing rate of SON vasopressin neurones (Khanna et al., 1993) and NPY injected into the PVN increases peripheral

vasopressin release (Leibowitz et al., 1988). However, the response of SON vasopressin neurones to i.c.v. administration of NPY is not known.

As NPY is co-released at sympathetic nerve terminals along with noradrenaline (NA) during sympathetic activation (Donoso et al., 1997), peripheral NPY concentration is increased by stressors (Krukoff et al., 1999). NPY from the periphery can readily enter the brain by diffusion across the blood-brain barrier (Kastin and Akerstrom, 1999). As oxytocin is a stress hormone in the rat (Lang et al., 1983), it can be hypothesized that stress-induced elevation of peripheral NPY may enhance activation of SON oxytocin neurones as a feedforward mechanism during stress. As NPY is a vasoconstrictor peripherally (Michel and Rascher, 1995), systemic administration of NPY may be expected to inhibit vasopressin neuronal activity. Hence, the effect of systemic administration of NPY on the electrical activity of SON oxytocin and vasopressin neurones was also studied.

3.2. Hypotheses

1. Central administration of NPY increases the electrical activity of SON oxytocin neurones in urethane-anaesthetized virgin rats and the response is attenuated in late-pregnant rats.
2. Systemic administration of NPY increases the electrical activity of SON oxytocin neurones in urethane-anaesthetized virgin rats *in vivo*.
3. Central administration of NPY increases while systemic administration of NPY decreases the electrical activity of SON vasopressin neurones in urethane-anaesthetized virgin rats *in vivo*.

3.2.1. Objectives

1. To study the effect of central administration of NPY on the electrical activity of SON oxytocin neurones in urethane-anaesthetized virgin and late-pregnant rats *in vivo*.
2. To study the effect of systemic administration of NPY on the electrical activity of SON oxytocin neurones in urethane-anaesthetized virgin rats *in vivo*.
3. To study the effect of central and systemic administration of NPY on the electrical activity of SON vasopressin neurones in urethane-anaesthetized virgin rats *in vivo*.

3.3. Background

The drive to obtain energy to sustain physiological well-being and the drive to reproduce successfully are the two instinctive urges essential for the survival of a species (Kalra and Kalra 2003). Neuropeptide Y (NPY) is one of the important neurochemical signals relaying information regarding these instincts (Kalra and Kalra 2003).

Neuropeptide Y was so-named because it is produced exclusively in neural tissue and has tyrosine (Y) residues at both ends (Kalra and Kalra 2003). It is considered to be the most abundant and widely distributed neuropeptide present in the mammalian central and peripheral nervous systems (Balasubramaniam, 1997).

NPY is a pleotrophic neuroendocrine signal (Horvath et al., 1997). As well as stimulating food intake (Clark et al., 1984), it is involved in the release of hypophysiotropic hormones (Kalra and Crowley, 1992; Kalra et al., 1992), inhibition of sexual behaviour (Clark et al., 1984) and a variety of autonomic activities (Gray and Morley, 1986). Central NPY markedly stimulates both nocturnal and diurnal feeding and drinking (Levine and Morley, 1984). NPY-induced food intake is suppressed by the opiate antagonist naloxone, and by the dopamine antagonist haloperidol (Levine and Morley, 1984). Based on the maximum quantity of food ingested following central administration of NPY, this peptide represents one of the most potent stimulators of feeding (Levine and Morley, 1984). However, NPY is not the only peptide involved in the upregulation of food intake because NPY knockout mice have a normal feeding phenotype and respond normally to fasting and refeeding (Erickson et al., 1996).

3.3.1. Hypothalamic source and distribution of NPY

The hypothalamic sources of NPY include the ARC NPY/AgRP neurones (Bai et al., 1985; Grove and Smith, 2003), NA neurones in the A1 and A2 cell group of the brainstem (Everitt et al., 1984; Sawchenko et al., 1985; Simonian and Herbison, 1997), amygdala, hippocampus (Allen et al., 1984; de Quidt and Emson, 1986), BNST, septum, LH, POA, peri- and paraventricular nuclei, DMN (Li et al., 1998a, b; Grove et al., 2003), mPVN and SON (Larsen et al., 1992a, b; Fetissov and Nicolaidis, 1998). However, the most abundant NPY innervation, reaching virtually the entire hypothalamus, arises from the ARC (Bai et al., 1985; Grove and Smith, 2003).

NPY neurones in the ARC co-express GABA (Horvath et al., 1997). NPY and GABA co-producing neurones are located in the dorsomedial ARC and non-GABAergic NPY neurones are located in the ventral ARC (Horvath et al., 1997). POMC neurones in the ARC express CART, a potent inhibitor of food intake (Elias et al., 1998; Kristensen et al., 1998; Lambert et al., 1998; Thim et al., 1998; Vrang et al., 1999). NPY and POMC

containing neurones in the ARC make synaptic contacts with each other (Csiffary et al., 1990). Many POMC neurones express Y_1R mRNA and protein (Broberger et al., 1997) and many NPY neurones in the ARC co-express AgRP, an endogenous MC4-R antagonist (Lu et al., 1994; Fong et al., 1997). Activation of AgRP-NPY neurones promotes feeding by activating NPY receptors and antagonizing MC4-R (Hahn et al., 1998). Activation of MC4-R decreases food intake, increases energy expenditure and decreases body weight (Vergoni et al., 2000; Wirth and Giraudo, 2001). Leptin decreases hypothalamic NPY mRNA expression (Stephens et al., 1995; Schwartz et al., 1996; Sahu, 1998a, b) and opposes orexigenic effects of NPY (Smith et al., 1996; Sahu, 1998a). NPY in turn antagonizes the anorectic effect of leptin (Erickson et al., 1996; Baskin et al., 1998). Leptin also decreases AgRP mRNA expression (Ebihara et al., 1999; Mizuno and Mobbs, 1999; Korner et al., 2001) and increases ARC POMC (Mizuno et al., 1998) and CART (Kristensen et al., 1998) mRNA expression enhancing its anorectic effect.

ARC-NPY neurones regulate the synthesis and release of many neurohormones such as GnRH, GHRH, somatostatin, CRH, TRH, vasopressin, oxytocin and dopamine (Kalra and Kalra 2003). They also innervate other hypothalamic nuclei that regulate appetite, sexual behaviour and energy expenditure (Kalra and Kalra 2003). As the ventromedial region of the ARC lacks an intact blood-brain barrier (Norsted et al., 2008), the ARC NPY neurones receive hormonal afferent messages from peripheral endocrine glands, leptin from adipocytes, ghrelin from stomach, steroids from gonads and adrenal cortex and cytokines from immune cells (Kalra and Kalra 2003). They also receive input signals from ascending neural pathways via brainstem catecholaminergic neurones and MCH neurones of the LH (Kalra and Kalra 2003).

3.3.2. Extra hypothalamic source of NPY: The brainstem

In the brain, 40% of NPY is synthesized in the ARC while the remainder is synthesized in the dorsovagal complex of the medulla (Bai et al., 1985; Chronwall, 1985; de Quidt and Emson, 1986). These brainstem NPY neurones project to other hypothalamic regions (Hokfelt et al., 1983b; Hokfelt et al., 1983a; Everitt et al., 1984). Especially, the C1 and A1 cell groups contain abundant NPY-ir (Sawchenko et al., 1985) and these neurones project to the SON (Shioda et al., 1992). NPY co-localizes with NA and adrenaline also in the LC (Everitt et al., 1984); however, the presence of NPY with NA in the A2 cell group is controversial (Harfstrand et al., 1987; Simonian and Herbison, 1997). NPY, indeed, is closely associated with NA and adrenaline in both the central and peripheral nervous system (Lundberg et al., 1985; Allen and Bloom, 1986; Gray and Morley, 1986; Morris et al., 1986). Under certain conditions, co-release of NPY and NA is important in amplifying or

restraining the postsynaptic response of hypothalamic target neurones (Kalra and Kalra 2003).

3.3.3. NPY receptors

NPY activates a heterogeneous population of at least six receptor subtypes, Y_1 - Y_6, all of which have been cloned except for the putative Y_3R (Inui, 1999; Fetissov et al., 2004). Y_1R mRNA is expressed in virtually all hypothalamic regions in the rat with the highest levels in the ARC and SON (Parker and Herzog, 1999). In the ARC, Y_1R-ip neurones are predominantly POMC neurones (Broberger et al., 1997; Fuxe et al., 1997).

Y_2R is an inhibitory autoreceptor in the presynaptic terminal where its activation inhibits the endogenous release of NPY (Zhang et al., 1997). Hence, central administration of Y_2R agonists decreases food intake (Leibowitz and Alexander, 1991). The presence of Y_2R has been established in many hypothalamic nuclei such as the ARC, mPOA, DMH, VMH, PVN, LHA and intensely in the SON (Gustafson et al., 1997; Parker and Herzog, 1999; Dumont et al., 2000). PYY_{3-36} is the agonist for Y_2R mediating satiety signal from the gut to the brain (Ueno et al., 2008).

Pancreatic polypeptide (PP), a peripheral appetite regulating peptide, is the natural ligand for the Y_4R (Bard et al., 1995; Lundell et al., 1995). However, high levels of Y_4R binding sites have been found in the mPOA, PVN, ARC and SON (Parker and Herzog, 1999; Dumont et al., 2000).

Y_5R mRNA has been localized in most of the hypothalamic sites including the PVN, LHA, ARC and SON (Gerald et al., 1996; Parker and Herzog, 1999). The highest level of Y_5R-ir was found in the magnocellular oxytocin and vasopressin neurones in the SON (Campbell et al., 2001; Wolak et al., 2003) and hence it is possible that NPY may have direct actions on the SON neurones to stimulate oxytocin and vasopressin release through the activation of Y_5R (Campbell et al., 2001).

The putative Y_3R has been characterized only in the NTS in the CNS (Lee and Miller, 1998) while the putative Y_6R is absent in rats, non-functional in the pig, guinea pig and humans and functional only in mouse and rabbit (Inui, 1999; Starback et al., 2000).

Y_1R and Y_5R subtypes have been implicated in the regulation of food intake (Criscione et al., 1998; Kalra et al., 1998) and they are widely distributed throughout the brain (Wolak et al., 2003). Surprisingly, Y_1 and Y_5 receptor knockout animals are not lean but develop late onset obesity (Kushi et al., 1998; Marsh et al., 1998; Pedrazzini et al., 1998; Kanatani et al., 2000). This could be attributed to redundancy of systems that control different functions, such as energy homeostasis, which could lead to compensatory changes during development (Eva et al., 2006).

I.c.v. administration of Y_1R and Y_5R antagonists suppresses NPY-induced feeding (48% and 40-50% reduction, respectively) and Fos-like immunoreactivity only in the mPVN (46% and 47% reduction, respectively) (Yokosuka et al., 1999; Yokosuka et al., 2001). Hence, it is likely that NPY receptor sites concerned with feeding behaviour reside selectively in the mPVN and Y_1R and Y_5R are either coexpressed or expressed separately in those target neurones that promote appetitive drive (Yokosuka et al., 2001).

3.3.4. NPY receptor signalling and downstream effects

NPY receptors belong to the GPCR superfamily. The YR gene family represents the largest and the most complex family of the GPCR superfamily for any known peptide (Blomqvist and Herzog, 1997). Binding of NPY to its receptors results in the inhibition of adenylate cyclase and activation of the phospholipase C/protein kinase C pathway (PLC/PKC) (Selbie et al., 1995; Parker et al., 1998; Leupen and Levine, 1999). Compounds that stimulate adenyl cyclase activity inhibit feeding while inhibition of cAMP synthesis tends to facilitate food intake (Chance et al., 2000). In accordance with this, intrahypothalamic administration of NPY inhibits hypothalamic adenyl cyclase activity and stimulates food intake in rats (Chance et al., 1989).

The perifornical hypothalamus (PFH) at the level of the caudal PVN is the most sensitive hypothalamic site for NPY-induced feeding (Stanley et al., 1993). In addition, NPY action on the PVN regulates feeding, thermogenesis and energy metabolism (Currie and Coscina, 1995). The CRH, CART and TRH neurones of the PVN have been shown to be downstream targets of NPY neurones (Heinrichs et al., 1998; Broberger, 1999; Kalra et al., 1999).

3.3.5. Mechanism of appetite regulation by ARC NPY neurones

A three-pronged interplay under the direction of NPY in the ARC-PVN axis initiates and extinguishes feeding behaviour (Kalra and Kalra 2003): 1. An increase in NPY and GABA release from the ARC NPY neurones sends information to higher brain centres to evoke feeding, while AgRP release from the ARC NPY neurones counteracts the tonic restraint from MC4 receptors to amplify appetite. 2. Increased NPY and GABA release within the ARC itself restrains POMC neurones causing a decreased release of alpha-MSH to diminish the tonic restraint on feeding. 3. After food intake, a gradual decrease in the release of NPY, GABA and AgRP in the PVN evoked by auto-feedback through Y_2R on NPY neurones in the ARC inhibits feeding.

During the time when a meal is anticipated, diminished leptin restraint from adipose tissue stimulates ghrelin pulses from the stomach. This results in activation of NPY, AgRP and GABA secretion, resulting in hunger. Consumption of a meal reverses this chain of

neural and hormonal events. Steady increase in leptin secretion reinstates tonic restraint on NPY ARC neurones, and along with decrease in ghrelin secretion, it results in appetite suppression (Kalra and Kalra 2003).

3.3.6. NPY and HPA axis

Central NPY activates the HPA axis in rats (Haas and George, 1987; Harfstrand et al., 1987; Wahlestedt et al., 1987; Suda et al., 1993). Central NPY stimulates CRH release in the median eminence (Haas and George, 1987, 1989), increases CRH mRNA expression in the pPVN (Suda et al., 1993) and increases plasma ACTH (Haas and George, 1989) and corticosterone (Harfstrand et al., 1987). NPY-induced increase in the circulating level of corticosterone might enhance glucose availability to ensure energy homeostasis during stress. Also, stress activates 36% of NPY neurones in the ARC, 33% in the NTS and 75% in the VLM (Krukoff et al., 1999).

3.3.7. Effect of NPY on SON neurones

Central NPY induces Fos in the SON (Niimi et al., 2001). Injection of NPY into the SON increases the firing rate of SON vasopressin neurones (Khanna et al., 1993) and NPY injected into the PVN increases peripheral vasopressin release (Leibowitz et al., 1988). NPY potentiates phenylephrine (α-1 adrenergic agonist) induced oxytocin and vasopressin release from perifused explants of the hypothalamo-neurohypophysial system (Kapoor and Sladek, 2001).

NPY excites only a small proportion (23%) of SON vasopressin neurones in pentobarbitone-anesthetized male rats (Day et al., 1985). Direct application of NPY onto the SON in pentobarbitone-anaesthetized male rats shows that NPY acts on Y_1R to excite vasopressin neurones but can also act on the presynaptic Y_2R to depress A1-vasopressin synaptic transmission (Khanna et al., 1993). Bath application of NPY excites SON neurones in hypothalamic slices (Sibbald et al., 1989). Application of NPY at a dose having virtually no direct effects produces a 5-fold increase in SON neurone responsiveness to NA, showing that NPY itself is only weakly excitatory to SON neurones but it markedly potentiates the excitatory effects of low concentrations of NA (Sibbald et al., 1989). It appears that NPY either directly activates SON neurones and/or modulates the response of these neurones to α-1 adrenergic stimulation (Willoughby and Blessing, 1987; Sibbald et al., 1989; Parker and Crowley, 1993; Kapoor and Sladek, 2001). The stimulatory effects of NPY on oxytocin and vasopressin release are mediated via activation of Y_1R and/or Y_5R, whereas activation of Y_2R may cause suppression of activity (Willoughby and Blessing, 1987; Khanna et al., 1993; Kapoor and Sladek, 2001).

3.3.8. Changes in NPY and NPY receptor expression in the CNS during pregnancy

During pregnancy, development of central leptin resistance around mid-gestation results in physiological hyperphagia despite high circulating levels of leptin (Grattan et al., 2007). Food intake increases by up to ca. 50% during pregnancy and it happens only during the dark phase (Cripps and Williams, 1975). Changes in NPY and its receptors expression during pregnancy have not yet been studied in the rats. However, in pregnant *Y_1R/LacZ* transgenic mice, NPY-ir is significantly increased in the PVN, decreased in the VMN and there is no change in the ARC (Oberto et al., 2003). In-situ hybridization study of specific hypothalamic areas shows that NPY mRNA content in the ARC significantly increases during pregnancy and lactation which might be partly responsible for the hyperphagia observed during these periods of increased energy demand (Garcia et al., 2003). However, studies that analysed total hypothalamic NPY mRNA by Northern blotting report that hypothalamic NPY mRNA does not change during mid- (Wilding et al., 1997) or late gestation (Ajala et al., 2001).

In pregnant *Y_1R/LacZ* transgenic mice, Y_1R gene expression is significantly increased in the PVN, ARC and medial amygdala and is reduced in the VMN (Oberto et al., 2002; Oberto et al., 2003). Administration of finasteride from day 12-17 of pregnancy prevented the increase in *Y_1R/LacZ* gene expression in the amygdala (Oberto et al., 2002). In virgin *Y_1R/LacZ* transgenic mice, long term treatment with progesterone or its 3α-reduced metabolite allopregnanalone increases Y_1R gene expression in the medial amygdala (Ferrara et al., 2001). These results suggest that neurosteroids that accumulate in the brain during pregnancy contribute significantly to alterations in NPY signalling in the CNS (Ferrara et al., 2001).

HPA and oxytocin responses to NPY are suppressed during pregnancy in the rat (Brunton et al., 2006a). Administration of naloxone, an opioid antagonist, restored the HPA response to central administration of NPY in late pregnancy (Bales et al., 2006b). In addition, suppression of NPY-induced oxytocin release and HPA activation in late pregnancy could be a result of increased effectiveness of GABAergic inhibitory input (Brunton et al., 2006a).

3.3.9. Peripheral effects of NPY

NPY and NA are co-released at the sympathetic neuro-effector junction (Pernow, 1988). However, acute stress and tonic sympathetic activation preferentially release NA, while NPY is released during prolonged and/or intense stress (Kuo et al., 2007). Cold water stress increases mean arterial pressure, heart rate, decreases mesenteric blood flow and increases mesenteric vascular resistance and a Y_1R antagonist inhibits up to 80% of the

vasoconstrictive effects of cold stress (Zukowska-Grojec et al., 1996). NPY has also been found in nonsympathetic neurones in several organs including the gastrointestinal tract, salivary glands, thyroid gland, pancreas, urinogenital system, airways and heart (Balasubramaniam, 1997). Peipheral actions of NPY include vasoconstriction, vascular remodelling, stimulating monocyte migration and activation, activation of platelets, angiogenesis and adipogenesis (Kuo et al., 2007). Peripheral NPY has also been implicated in stress-induced augmentation of diet-induced obesity by increasing angiogenesis and adipogenesis and reducing lipolysis (Kuo et al., 2007).

3.4. Methods

3.4.1. Experiment 1: Effect of central administration of NPY on the electrical activity of SON oxytocin and vasopressin neurones in urethane-anaesthetised virgin and pregnant rats in vivo.

Urethane-anaesthetized virgin and pregnant (19-21d of pregnancy) rats were fixed with an i.c.v. cannula and an i.v. cannula on the day of the experiment. The SON and neural stalk were exposed via the ventral transpharyngeal surgery. Stimulating and recording electrodes were placed in position. Once SON neurones were identified and oxytocin neurones and vasopressin neurones were differentiated, 2µl aCSF or 5µg/2µl of NPY was administered i.c.v. after an initial basal recording of firing rate for 10min.

Groups:

a. Oxytocin neurones
    i. Virgin aCSF: n = 5
    ii. Pregnant aCSF: n = 4
    iii. Virgin NPY: n = 5
    iv. Pregnant NPY: n = 6
b. Vasopressin neurones:
    i. Virgin NPY: Non-phasic: n = 4

3.4.2. Experiment 2: Effect of systemic administration (20µg per rat in 100µl saline; i.v) of NPY on the electrical activity of SON oxytocin and vasopressin neurones in urethane-anaesthetised virgin rats in vivo.

Groups:

a. Oxytocin neurones: n = 5
b. Vasopressin neurones:
    i. Phasic: n = 3
    ii. Non-phasic: n = 6

3.5. Results

3.5.1. Body weight

As expected, the pregnant groups had significantly higher body weight than the virgin groups (Fig. 3.1).

3.5.2. Basal firing rate of SON oxytocin neurones

The basal firing rate did not differ within virgin and pregnant groups. However, the basal firing rate of oxytocin neurones in the pregnant groups was significantly higher than the respective virgin groups (Fig. 3.2).

3.5.3. Effect of central administration of NPY on the electrical activity of SON oxytocin neurones in virgin rats

Central administration of NPY (5µg/2µl aCSF; i.c.v) excited all five SON oxytocin neurones tested (Fig. 3.3) while a vehicle injection did not alter the basal firing rate (Fig. 3.4). In the virgin NPY group (n=5 cells/rats), the basal rate of 3.5 ± 1.1 spikes/s was increased by 1 ± 0.45 spikes/s 1min after NPY. It remained elevated and returned to basal by 40min on average. The average change in firing in rate 0-30min after NPY was significantly higher than that during the basal period (Fig. 3.5). In the virgin aCSF group (n=4), there was no change in the firing rate after aCSF administration. The mean increase in firing rate 0-30min after NPY in the virgin NPY group was significantly higher than the change in firing rate 0-30min after aCSF in the virgin aCSF group (Fig. 3.6).

After NPY there was an overall increase in the number of spikes occurring within 0-0.5s of the preceding spike compared to the basal period as shown by the interspike interval histogram before and after NPY administration (Fig. 3.7a). The descending slope of the histograms fitted well with exponential curves (Fig. 3.7b). There was no apparent change in the shape in the hazard plots for 10min before and after NPY (Fig. 3.8). The ratio of peak early (<0.07s) to mean late (0.2 – 0.3s) hazard before and after NPY did not differ from each other (Fig. 3.9).

3.5.4. Effect of central administration of NPY on the electrical activity of SON oxytocin neurones in pregnant rats

In the pregnant aCSF group (n=4), the average basal firing rate was 6.6 ± 0.6 spikes/s and the vehicle injection (2µl aCSF; i.c.v) did not alter the firing rate significantly (e.g. Fig. 3.10). Central administration of NPY (5µg/2µl aCSF; i.c.v) excited all six SON oxytocin neurones tested in late pregnant rats (e.g. Fig. 3.11). On average, the basal firing rate of 6.5 ± 0.8 spikes/s was increased by 1.2 ± 0.3 spikes/s 9min after NPY injection. The rate remained elevated until returning to basal again by 25min. The mean increase in firing rate 0-30min after NPY compared to the change in firing rate during the basal period was

significantly greater. The average firing rate 0-30min after NPY in the pregnant NPY group was significantly higher than the rate 0-30min after aCSF injection in the pregnant aCSF group (Fig. 3.12) but it was not different from the firing rate observed in the virgin NPY group (Fig. 3.13).

3.5.5. Effect of systemic administration of NPY on firing rate of SON oxytocin neurones in virgin rats

Systemic administration of NPY (20µg; i.v) excited four of five SON oxytocin neurones tested (E.g. Fig. 3.14) while one neurone did not respond. On average, the basal firing rate of 2.5 ± 0.95 spikes/s was increased by 1.4 ± 0.6 spikes/s 30s after NPY (n=5). The mean increase in firing rate 0-5min after NPY was significantly higher than that during the basal period (Fig. 3.15).

3.5.6. Effect of central administration of NPY on the firing rate of SON vasopressin neurones in virgin rats

The effect of central administration of NPY was observed in four non-phasic and one phasically firing SON vasopressin neurones. Among the four non-phasic vasopressin neurones, 2 were excited, 1 was inhibited and 1 did not respond. On average, the basal firing rate was 6.6 ± 1.2 spikes/s and it was not altered by central NPY administration (Fig. 3.16). There was also little alteration (a slight inhibition) in the firing of the phasic vasopressin neurone after central NPY. The activity quotient (0.06) was unaltered by NPY; frequency within bursts was slightly reduced from 4.2 spikes/s to 4.04 spikes/s 0-5min after NPY; mean interburst interval increased from 3.9s to 5.7s 0-5min after NPY. There was no change in the interspike intervals before and after NPY (Fig. 3.17a). The distal tails of the histogram fitted well with exponential curves leaving the intervals before 0.2s which is a characteristic for vasopressin neurones (Fig. 3.17b). NPY administration did not alter the shape of the hazard plot (Fig. 3.18).

3.5.7. Effect of systemic administration of NPY on the firing rate of SON vasopressin neurones in virgin rats

The effects of systemic administration of NPY were observed in eight non-phasic and three phasically firing SON vasopressin neurones. Among the eight non-phasic vasopressin neurones, 7 were inhibited (e.g. Fig. 3.19) and 1 did not respond. On average, the basal firing rate of 7 ± 0.99 spikes/s was reduced by 2.4 ± 1.8 spikes/s 1min after NPY (n=6). The firing rate 0-5min after NPY was not different from the basal period (Fig. 3.20).

Systemic NPY administration transiently inhibited all three phasic vasopressin neurones tested (e.g. Fig. 3.21). However, the formal measures of activity of the 3 cells did not show significant effects (Fig. 3.22, 3.23 and 3.24).

3.6. Discussion

3.6.1. Central NPY-induced excitation of SON oxytocin neurones

This study reports for the first time that central administration of NPY increases the electrical activity of SON oxytocin neurones in female rats *in vivo*. There are other electrophysiological studies reporting activation of only SON vasopressin neurones by direct application of NPY in male rats *in vivo* (Day et al., 1985; Willoughby and Blessing, 1987) or *in vitro* (Sibbald et al., 1989).

Central administration of NPY did not alter the shape of the hazard plot and this is supported by the lack of change in the ratios of peak early to mean late hazard before and after NPY. Hence, central NPY administration evidently did not alter the intrinsic properties [such as resting membrane potential, input resistance, depolarizing afterpotential (Stern and Armstrong, 1996)] of the SON oxytocin neurones. The positive shift in the hazard function without a change in the shape of the hazard plot indicates an increase in the synaptic drive and/or a sustained depolarization upon which continued/increased synaptic drive is superimposed (Brown et al., 2008). It is possible that central NPY potentiates the effects of excitatory noradrenergic inputs from the brainstem onto the SON as shown in studies *in vitro* (Sibbald et al., 1989).

As central administration of NPY induces Fos activation in SON oxytocin neurones and increases peripheral oxytocin release (Brunton et al., 2006a), this study reinforces the point that Fos activation most often accompanies an increase in neuronal electrical activity which in turn, in neurosecretory cells, is accompanied by the release of the neuropeptides.

3.6.2. Physiological importance of central NPY-induced excitation of SON oxytocin neurones

NPY-induced excitation of oxytocin neurones and the accompanying peripheral release of oxytocin might be physiologically important for at least three reasons.

1. Stress-induced activation of oxytocin neurones via NPY neurones: Stressors activate NPY neurones in the ARC and brainstem (Krukoff et al., 1999) and oxytocin is also a stress hormone in the rat (Lang et al., 1983). Hence, stress-induced activation of NPY neurones might potentiate stress-induced activation of oxytocin neurones. These physiological modulations of oxytocin neurones by NPY might be possible because the ARC and the brainstem neurones, where NPY is expressed in abundance (Chronwall, 1985), project directly to the SON (Leng et al., 1988; Shioda et al., 1992) (though it is not known whether ARC-NPY neurones particularly project to the SON) and SON neurones strongly express NPY receptors (Parker and Herzog, 1999; Campbell et al., 2001; Wolak et al., 2003).

As the function of neuropeptides such as NPY is often to amplify and reinforce the actions of other neurotransmitters regulating important physiological processes (Urban et al., 2006), in addition to other neuropeptides/pathways, NPY might also increase oxytocin neuronal activity and peripheral oxytocin release to ensure homeostasis during stress.

As peripheral oxytocin in itself increases glucose availability and uptake by increasing glucagon and insulin release, respectively (Altszuler et al., 1992; Lee et al., 2008), NPY-induced oxytocin release might be a valuable mechanism to enhance glucose availability during increased energy demand.

2. Activation of SON oxytocin neurones by NPY during/after food intake: NA/NPY neurones in the A1 and A2 cell groups are activated by food intake and associated stimuli such as gastric distension, vagal stimulation, gut peptides (e.g. CCK) (Monnikes et al., 1997; Willing and Berthoud, 1997; Rinaman et al., 1998; Conde et al., 1999; Krukoff et al., 1999; Ohiwa et al., 2006; Appleyard et al., 2007). Activation of NA neurones in the A2 cell group results in the excitation of SON oxytocin neurones via excitatory NA projections (Raby and Renaud, 1989; Onaka et al., 1995a; Buller and Day, 1996). Hence, central NPY administration might presumably resemble NA/NPY discharge from the axon terminals at the SON in response to stimuli such as food intake and associated CCK release.

3. Activation of SON oxytocin neurones by NPY during food anticipation: NPY is a orexigenic neuropeptide and anticipation for food upregulates NPY in the ARC (Drazen et al., 2005). However, ARC-NPY neuronal projections to the SON have not been established. In addition, SON oxytocin neurones are not activated during food anticipation (Johnstone et al., 2006). Activation of SON oxytocin neurones by NPY during food anticipation appears irrelevant because, physiologically, oxytocin as a promoter of natriuresis needs to be released after food intake not during food anticipation.

4. Activation of SON oxytocin neurones by NPY during food deprivation: Oxytocin from the magnocellular neurones can enter the hypothalamo-hypophysial portal blood to potentiate basal or CRH/AVP (Arginine vasopressin)-induced ACTH and subsequent corticosterone secretion (Makara et al., 1982; Lang et al., 1983; Gibbs, 1985, 1986; Engelmann et al., 2004). Food deprivation-induced upregulation of ARC NPY (Kalra and Kalra, 2003) might activate oxytocin neurones and increase oxytocin release so as to potentiate CRH-induced ACTH secretion. The resulting increase in endogenous glucocorticoids will ensure mobilization of glucose to meet the energy demand until food is available (Olefsky and Kimmerling, 1976). However, it is not known whether ARC-NPY neurones project to the SON.

3.6.3. Physiological effects of central NPY and oxytocin

1. Anxiolysis: The drive for energy is an absolute essential for the survival of a species. However, procuring food during a meal time could be an arduous task for animals in the wild, resulting in anxiety. Food restriction increases anxiolytic-like behaviour (Inoue et al., 2004) suggesting that anxiety associated with anticipation/absence of food is alleviated naturally. It is likely that an endogenous mechanism for anxiolysis is in place through NPY upregulation during meal time because central NPY acts as an anxiolytic (Heilig and Murison, 1987a, b; Heilig et al., 1989; Heilig et al., 1992; Baraban et al., 1997; Bannon et al., 2000; Stogner and Holmes, 2000; Thorsell et al., 2000; Kask et al., 2001a; Kask et al., 2001b; Redrobe et al., 2002). GABA and its receptor agonists increase feeding and induce anxiolysis in animal models of anxiety (Lopes et al., 2007). As NPY is co-localized with GABA in several limbic structures (Horvath et al., 1997; Pu et al., 1999), some of the central actions of NPY, like anxiolysis, might involve interaction with GABAergic transmission (Pu et al., 1999).

Central oxytocin also is anxiolytic (Mantella et al., 2003; Amico et al., 2004; Ring et al., 2006). NPY potentiates NA-induced oxytocin release from hypothalamo-hypophysial explants (Kapoor and Sladek, 2001). If NPY-induced excitation of oxytocin neurones is accompanied by dendritic release of oxytocin, oxytocin and NPY might complement each other in their anxiolytic effects. Anxiolysis during the postprandial period might also enhance the feeling of satiety. Hence, anxiolytic effects of NPY and oxytocin could be beneficial not only during stress but also around the meal time making the entire process of feeding pleasurable.

2. Appetite regulation: As NPY is one of the most potent appetite stimulants known, check-points like oxytocin, through its central anorectic actions (Olson et al., 1991a, b, c), might be needed even during normal feeding to balance the potent orexigenic effect of NPY so as to regulate appetite and meal size, and consequently body weight.

It is interesting that Y_1, Y_2 and Y_5 receptor knockout mice are not lean but develop late onset obesity (Kushi et al., 1998; Marsh et al., 1998; Pedrazzini et al., 1998; Kanatani et al., 2000) similar to oxytocin receptor deficient mice (Takayanagi et al., 2008). However, it is not known whether NPY and oxytocin receptor modulation depend on each other in this context.

3. Regulation of behaviour: In addition to appetite regulation and anxiety alleviation, central actions of NPY and oxytocin have been implicated in diverse social behaviours (van Wimersma Greidanus et al., 1986; Carter et al., 2008; Karl et al., 2008; Veenema and Neumann, 2008), behavioural adaptation to stress (Thorsell et al., 1999; Ebner et al., 2005),

learning (Argiolas and Gessa, 1991; de Wied et al., 1993; Gulpinar and Yegen, 2004; Eaton et al., 2007), memory (Argiolas and Gessa, 1991; de Wied et al., 1993; Cleary et al., 1994; Gulpinar and Yegen, 2004; Fang et al., 2008) and reward processes (Argiolas and Gessa, 1991; de Wied et al., 1993; Brown et al., 2000b; Marazziti and Catena Dell'osso, 2008). Central oxytocin might also enhance the memory related to food and feeding behaviour (Neumann, 2008). NPY plays a role in the motivation to eat and in food anticipatory responses (Flood and Morley, 1991; Drazen et al., 2005). Hence it is likely that central NPY and oxytocin interact with each other to modulate complex behavioural patterns.

3.6.4. Central NPY-induced excitation of SON oxytocin neurones in pregnant rats: Discrepancy in the basal activity

The basal activity of SON oxytocin neurones during late gestation is expected to be low to prevent the risk of preterm labour and to preserve the oxytocin stores that are required for impending parturition. However, the basal firing rate of SON oxytocin neurones in pregnant rats observed in this study was higher than that reported earlier (Douglas et al., 1995; Brunton et al., 2006b). This might be the result of influence of the day of gestation of the rats used (19-21d in this study *vs.* 20-21d and 21d by the other studies) (Douglas et al., 1995; Brunton et al., 2006b). However, it is also possible that the present measurement reflects the small sample of cells (n=5).

3.6.5. Central NPY-induced excitation of SON oxytocin neurones in pregnant rats: Discrepancy in NPY-induced increase in activity

Surprisingly, the SON oxytocin neurone electrical activity in response to central NPY was not attenuated in urethane anaesthetized late-pregnant rats. This conflicts with the attenuated Fos induction and oxytocin secretory response previously observed in conscious late-pregnant rats following central NPY (Brunton et al., 2006a).

3.6.5.1. Discrepancy: Due to anaesthesia and surgery?

As autonomic and neuroendocrine responses are influenced by surgery and anaesthetics, different results may be obtained when comparing studies in conscious and anesthetized animals (Watanabe et al., 2004). For example, electrical stimulation of the PVN produces excitatory responses in renal sympathetic nerve activity in unanaesthetized rats but not in anaesthetized rats (Kannan et al., 1989). Hence, the opposing results for the effects of i.c.v. NPY observed in conscious and anaesthetized late-pregnant rats might be due to the effect of anaesthetic and the complex surgical procedure followed to expose the SON in the present study.

Urethane preserves the physiological functioning of SON oxytocin neurones as it does not affect the milk ejection reflex (Leng and Dyball, 1991). However, intraperitoneal

injection of urethane results in the movement of water from the circulation into the peritoneal fluid resulting in plasma hyperosmolality (Van Der Meer et al., 1975). SON oxytocin neurones are osmosensitive (Mason, 1980) and hyperosmotic stimuli increase their activity (Leng et al., 2001). Although plasma osmolality is reduced during pregnancy (Brunton et al., 2008), urethane, and hence the hyperosmolality-induced increase in firing rate in anaesthetised pregnant rats might be the cause of lack of attenuation of oxytocin neurone activity upon central NPY.

However, in a similar study of urethane anaesthetized virgin and late-pregnant rats, the electrophysiological response of the electrical activity of SON oxytocin neurones to systemic administration of IL-1β was attenuated in the pregnant rats (Brunton et al., 2006b). Lack of attenuation following NPY in the present study indicates that the immune challenge and orexigenic stimuli might be processed differently by the inputs to the oxytocin system.

3.6.5.2. Discrepancy: Due to lack of feeding?

The differences observed between conscious and anaesthetized rats in SON oxytocin neurone responses upon central administration of NPY could also be attributed to absence of food and water intake after central NPY and absence of food/consciousness-related sensory inputs as a result of anaesthesia.

Following central administration of NPY, food and water intake significantly increased in conscious virgin and late pregnant rats (Brunton et al., 2006a). However, absence of availability of food and water in anaesthetized rats following NPY might alter the peripheral input signals to the oxytocin system. For example, the stimulatory effect of NPY on the HPA axis in rats with free access to food after NPY was stronger than the effect observed in rats that were fasted after NPY treatment (Hanson and Dallman, 1995). Fos expression in PVN and SON neurones after central NPY followed by food intake was much greater than that after NPY without food (Li et al., 1994). In addition, oropharyngeal stimulation during food intake and gastric distension during/after food intake activates, through vagal afferents, brainstem neurones (Rinaman et al., 1998; Liu et al., 2004; Sabbatini et al., 2004; van de Wall et al., 2005; Huo et al., 2007) and the SON receives a substantial excitatory noradrenergic innervation from the brainstem (Alonso and Assenmacher, 1984). Absence of these peripheral feeding-related signals in anaesthetized rats might alter the oxytocin electrophysiological response.

3.6.5.3. Discrepancy: Due to lack of inputs?

In addition, the SON oxytocin system is well connected to limbic structures (Oldfield et al., 1985; Oldfield and Silverman, 1985) and central oxytocin modulates social and feeding behaviours (Olszewski et al., 2007; Amico et al., 2008). Hence, lack of consciousness during anaesthesia resulting in diminished sensory inputs to the oxytocin system might also contribute to the differences in oxytocin neurone responses observed in conscious and anaesthetized animals.

3.6.5.4. Discrepancy: Due to enhanced endogenous NPY activity?

The number of neurones expressing NPY Y_1R and the amount of Y_1R within the magnocellular neurones are increased with hypertonic saline ingestion or water deprivation (Urban et al., 2006). In euhydrated animals, 30 and 36% of oxytocin and vasopressin neurones, respectively, coexpressed Y_1R whereas these values were increased to 52 and 63%, respectively, in salt-loaded animals (Urban et al., 2006). Under normotensive conditions, the expression of NPY-ir within the magnocellular divisions of the hypothalamus is primarily within the fibres in the PVN and SON and there are few NPY-ir neurones within the magnocellular system (Urban et al., 2006). Subjecting animals to osmotic stressors, either water deprivation or replacing the drinking water with hypertonic saline, significantly increases NPY mRNA and peptide expression within the PVN and SON (Larsen et al., 1992b; Larsen et al., 1993). In addition, haemorrhage increases NPY gene expression within the C1 and A1 groups in the medulla (Chan and Sawchenko, 1998). Hence, the NPY neurones projecting to, and within, the hypothalamus are activated under conditions of hyperosmolarity, haemorrhage or decreased blood pressure (Urban et al., 2006). All these conditions prevail in the animal model used in this study: urethane results in hyperosmolarity and the ventral transpharyngeal surgery to expose the SON results in a certain degree of haemorrhage and decreased blood pressure. This is again compounded by the lack of water intake under anaesthesia. These conditions of altered water homeostasis and increased endogenous NPY activity might also contribute to the unattenuated oxytocin neurone response observed in anaesthetized pregnant rats upon acute central NPY administration in this study.

3.6.6. Systemic NPY-induced excitation of SON oxytocin neurones

Systemic administration of NPY also increased the electrical activity of SON oxytocin neurones in this study. The mode of action of peripheral NPY on oxytocin neurones is not known; however, NPY can enter the brain by diffusion across the blood-brain barrier (Kastin and Akerstrom, 1999).

3.6.7. Regulation of physiological processes by systemic NPY and oxytocin

1. Stress: Stressors increase peripheral NPY levels (Krukoff et al., 1999). In addition, NPY is co-released at sympathetic nerve terminals along with NA during sympathetic activation (Donoso et al., 1997). Hence, physiologically, peripheral stress-induced NPY release might potentiate oxytocin secretion during the stress response.

2. Adiposity: Chronic increase in peripheral levels of NPY results in adiposity (Kuo et al., 2007), on the contrary, oxytocin negatively modulates adipogenesis (Elabd et al., 2008). Hence, peripheral oxytocin might be important to neutralise the adipogenic effect of peripheral NPY to ensure constancy of body weight even during adverse stressful conditions.

3. Cardiovascular regulation: Peripheral NPY and oxytocin are also opposite in their effects in cardiovascular regulation. Peripheral oxytocin results in a dose-related biphasic change in mean arterial pressure which consists of an initial pressor effect accompanied by bradycardia and a decrease in cardiac output, followed by a more prolonged fall in mean arterial pressure accompanied by an increase in cardiac output (Petty et al., 1985). Overall, oxytocin causes a long-term decrease of blood pressure in rats (Petersson et al., 1996). On the other hand, NPY has a vasopressor effect by direct vasoconstriction of blood vessels and by potentiating NA-evoked responses (Grundemar and Hakanson, 1993). Hence, oxytocin release might be essential to neutralise or reduce vasopressor effects of NPY during stress or sympathetic activation. It is noteworthy that oxytocin is capable of inducing the release of ANP from the heart (Haanwinckel et al., 1995; Gutkowska et al., 1997) which exerts a relaxing effect on vasculature and antagonizes the contractions induced by norepinephrine (Waeber et al., 1990). These effects of ANP are also opposite to the effects of NPY on the vasculature (Grundemar and Hakanson, 1993). The opposing roles of these peptides may have important implications in cardiovascular physiology.

4. Natriuresis: Peripheral oxytocin is natriuretic either directly or indirectly through ANP (Conrad et al., 1993; Haanwinckel et al., 1995). Similarly, peripheral NPY is also natriuretic (Allen et al., 1985; Bischoff et al., 1996; Bischoff et al., 1998a, b; Bischoff and Michel, 1998). In addition, NPY significantly increases plasma ANP in a dose-related manner in normally-hydrated rats as well as in water-loaded rats, indicating that an increase of ANP release may be responsible for NPY-induced natriuresis (Baranowska et al., 1987). Hence, peripheral NPY and oxytocin interaction might also be important for water and electrolyte homeostasis.

3.6.8. NPY-induced changes in the electrical activity of SON vasopressin neurones

Central NPY did not have much effect on the activity of SON vasopressin neurones in this study. Injection of NPY into the SON increased the firing rate of SON vasopressin

neurones (Khanna et al., 1993). A conclusive result might have been obtained had the response to central NPY been studied in more vasopressin neurones. However, the responses of vasopressin neurones to various stimuli, such as secretin, CCK and leptin, have always been mixed with a percentage of neurones being excited or inhibited and some other not responding (See secretin/CCK/leptin chapters).

Systemic administration of NPY inhibited the three SON vasopressin neurones tested. Systemic NPY is a vasoconstrictor through its postsynaptic activation of NA receptor mechanisms in the sympathetic nerve terminals (Lundberg et al., 1990). Hence, the acute inhibition of vasopressin neurones could be possibly due to increase in the blood pressure following NPY-induced vasoconstriction. As vasopressin is also a vasoconstrictor, inhibition of vasopressin neuronal activation by systemic NPY could possibly be a feedback mechanism preventing excessive vasoconstriction.

As NPY rapidly diffuses across the blood-brain barrier, systemic NPY might also alter the electrical activity of SON vasopressin neurones either by direct action on the NPY receptors in the neurones or indirectly via presynaptic regulation of NA release on to the SON. Direct application of NPY onto the SON in pentobarbitone-anaesthetized male rats showed that NPY acts on Y_1R to excite vasopressin neurones, but can also act on the presynaptic Y_2R to depress A1 to vasopressin neurone synaptic transmission (Khanna et al., 1993). The inhibitory effect of systemic NPY on vasopressin neurones observed in this study suggests the second possibility.

3.7. Conclusion

Centrally administered NPY increases the electrical activity of SON oxytocin neurones in urethane-anaesthetized virgin rats, with similar effects in late pregnant rats. These findings contrast with studies in conscious rats in which i.c.v NPY activated oxytocin neurones in virgin, but not late pregnant rats. Systemic administration of NPY also increased the firing rate of SON oxytocin neurones in virgin rats (pregnant rats were not tested). Oxytocin neurone excitation induced by NPY may be relevant during stress responses. In addition, it is likely that peripheral/central NPY and oxytocin interact with each other to regulate complex physiological processes such as appetite, stress regulation, anxiety, behaviour, cardiovascular regulation and water/electrolyte homeostasis.

CHAPTER IV

LEPTIN

4.1. Introduction

Leptin, synthesised in adipose tissue, is a centrally-acting anorexigenic hormone. Leptin acts in the hypothalamus to reduce food intake and increase energy expenditure (Campfield et al., 1995; Pelleymounter et al., 1995).

Most supraoptic nucleus (SON) neurones are strongly immunoreactive for short and long isoforms of leptin receptor (Hakansson et al., 1998). Oxytocin and vasopressin mRNA expression in the PVN were down-regulated by 48h fasting in mice and were restored by leptin (Tung et al., 2008). The effect of bath application of leptin was predominantly inhibitory on SON neurones *in vitro* (Honda et al., 2002) and intracerebroventricular (i.c.v) administration of leptin did not induce STAT3 activation in the SON in rats (Hubschle et al., 2001) and did not induce Fos expression in the mPVN and SON (Yokosuka et al., 1998).

Systemic leptin administration increases Fos expression in the pPVN, but not in oxytocin neurones (Elias et al., 2000). Systemic leptin did not induce pSTAT3 or Fos (Celine Caquineau, *personal communication*) but increases nuclear STAT5 in the SON (Mutze et al., 2007). As the activation of STAT pathway by leptin may indicate either excitation or inhibition of SON neurones, it is not clear whether systemic leptin excites or inhibits the electrical activity of SON oxytocin and vasopressin neurones *in vivo*. However, leptin, a long-term satiety signal, may not have an immediate effect on SON neurones.

Pregnant females do not show suppression of food intake in response to exogenous leptin (Mounzih et al., 1998; Johnstone and Higuchi, 2001; Ladyman and Grattan, 2004; Lecklin et al., 2005). Moreover, an increase in plasma leptin levels was observed especially during the latter half of pregnancy, when maternal and food consumption markedly increased (Kawai et al., 1997). This paradoxical increase in leptin suggests that pregnancy is a physiological state of leptin resistance. As oxytocin neurone responses to various stimuli are attenuated during pregnancy (Douglas et al., 2007), it was hypothesized that leptin-induced effects on SON oxytocin neurones are suppressed in pregnant rats (Fig. 4.1). However, it is not known whether the leptin-induced effects in SON oxytocin neurones are modified by pregnancy, which might contribute to resistance to anorectic effects of leptin.

Fasting reduces circulating leptin concentration (Maffei et al., 1995). Fasting for 24h decreases plasma leptin level significantly in both pregnant and non-pregnant rats (Ladyman and Grattan, 2004) and fasting for 3 days significantly decreases leptin transport across the blood-brain barrier in mice (Kastin and Akerstrom, 2000). It may be hypothesized that leptin-induced excitatory effects will be more pronounced after fasting as fasting-induced

reduction in endogenous levels of leptin will increase hypothalamic sensitivity to exogenous leptin. On the other hand, fasting may reduce the activity of SON neurones, so that natriuresis could be avoided by reducing peripheral release of oxytocin to any stimuli including leptin as fasting reduces sodium and water intake. In addition, if increased firing rate of SON oxytocin neurones in unfasted rats in response to leptin infusion leads to central release of oxytocin, this is expected to reduce food intake and salt appetite in rats fed *ad libitum* (Blackburn et al., 1993). In fasted rats, suppressed central release of oxytocin in response to leptin is expected to permit increased appetite.

4.2. Hypothesis

1. Systemic leptin, a long-term satiety signal, does not affect the electrical activity of SON oxytocin and vasopressin neurones in virgin rats.
2. SON oxytocin neurones are resistant to the effects of systemic leptin during late pregnancy.
3. Leptin-induced effects in the SON oxytocin neurones are either positively (due to increased sensitivity) or negatively (to prevent natriuresis) altered by fasting in virgin and late-pregnant rats.
4. Leptin-induced changes in the electrical activity of SON oxytocin neurones in fasted rats are dose dependent.

4.2.1. Objectives

1. To study the electrophysiological responses of SON oxytocin neurones to systemic administration of leptin *in vivo* in unfasted/fasted virgin/pregnant rats.
2. To study whether leptin-induced changes in electrical activity of SON oxytocin neurones in fasted rats are dose dependent.
3. To study the electrophysiological responses of SON vasopressin neurones to systemic administration of leptin *in vivo*.

4.3. Background

4.3.1. Leptin and its receptors

Leptin is a hormone derived from adipose tissue that primarily acts in the hypothalamus to control body fat levels by regulating appetite and energy metabolism (Campfield et al., 1995; Halaas et al., 1995; Pelleymounter et al., 1995). Leptin secretion correlates with the amount of fat in the body, therefore the circulating level of leptin indicates the current body fat store (Maffei et al., 1995; Considine et al., 1996). Plasma leptin concentration follows a circadian rhythm. In rats, it rises to maximal values during the night after they start eating (Merino et al., 2008).

Leptin mediates its effects through its receptors**.** Five leptin receptor subtypes (Ob-Ra to Ob-Re) have been identified (Lee et al., 1996; Fei et al., 1997; Leibel et al., 1997; Friedman, 1998). Ob-Ra, a predominant short form of leptin receptor is capable of leptin-mediated signalling but much less effectively than the full-length receptor, Ob-Rb (Murakami et al., 1997). Short forms of leptin receptors could function as specific transport systems for leptin, as they are present in high amounts in the choroid plexus and brain microvessels (Tartaglia et al., 1995; Lee et al., 1996; Guan et al., 1997; Bjorbaek et al., 1998). The long form, Ob-Rb, is the functional receptor through which leptin mediates its effects and is widely distributed in the brain including the ARC, VMH, PVN, DMH, SON, lateral POA, mPOA and SCN (Hakansson et al., 1996; Mercer et al., 1996; Fei et al., 1997; Zamorano et al., 1997; Bjorbaek et al., 1998; Elmquist et al., 1998; Mercer et al., 1998; Shioda et al., 1998; Yarnell et al., 1998). The secretory form, Ob-Re, which is spliced in ahead of the transmembrane domain, might be a soluble binding protein for leptin (Lee et al., 1996). The other short forms could behave as functional antagonists by sequestering leptin and preventing its binding to the Ob-Rb subtype (White et al., 1997). Plasma leptin crosses the blood-brain barrier via a receptor mediated transport system (Banks et al., 1996; Golden et al., 1997; Hileman et al., 2002).

4.3.2. Leptin action in hypothalamus

The binding of leptin to its receptor results in a rapid activation of intracellular JAK2. JAK2 tyrosine phosphorylates STAT3 proteins. pSTAT3 then dissociates and forms dimers in the cytoplasm, finally translocating to the nucleus to regulate gene transcription. STAT3 activation is likely a crucial component in regulation of body weight by leptin (Bjorbaek and Kahn, 2004). Studying pSTAT3 activation to monitor leptin-induced effects is more appropriate than Fos studies because Fos immunoreactivity colocalizes only in a subset of all leptin-responsive STAT3 nuclei (Hubschle et al., 2001). However, Fos almost always

indicates neuronal excitation [there are exceptions; (Sabatier et al., 2003a)] while pSTAT3 activation indicates either leptin-induced excitation or inhibition (Hubschle et al., 2001).

Systemic leptin induces Fos expression in the hypothalamus (Woods and Stock, 1996; Elmquist et al., 1997) including in the PVN, DMH, VMH and ARC (Elias et al., 2000). In the ARC, leptin-induced Fos is expressed in CART, dynorphin and neurotensin expressing neurones, while in the VMH Fos is seen in dynorphin neurones. Fos and Ob-Rb mRNA double-labelled neurones are found in the ARC, VMH and DMH (Elias et al., 2000).

Leptin decreases food intake more potently when microinjected directly into the ARC and hence the ARC is a primary hypothalamic site of the satiety effect of leptin (Satoh et al., 1997a). About 40% of ARC neurones in Wistar rats are inhibited by bath application of leptin at 10^{-8} to 10^{-6} M (Rauch et al., 2000). Leptin decreased input resistance and inhibited evoked excitatory postsynaptic currents in ARC neurones (Glaum et al., 1996). Bath application of 10^{-10} M leptin inhibited about 60% and excited only 4% of the ventromedial ARC (vmARC) neurones where leptin receptors are co-localized with NPY (Nagamori et al., 2003). On direct bath application of 10^{-11} – 10^{-10} M leptin, glucose sensitive neurones of the ARC showed a marked dose-dependent decrease in activity after long latency (Shiraishi et al., 1999). In contrast, leptin increases the frequency of action potentials in the anorexigenic POMC neurones (Cowley et al., 2001).

The satiety effect of leptin is also mediated via the VMH (Satoh et al., 1997b). VMH-lesioned rats overproduce leptin but cannot respond to it (Satoh et al., 1997b). Glucose-receptive neurones in the VMH were hyperpolarized by bath application of leptin (4-50nM) through activation of ATP-sensitive potassium channels (Spanswick et al., 1997). However, in an *in-vivo* study, microiontophoretic administration of leptin increased the activity of glucose responsive and non-glucose responsive neurones in the VMH (Shiraishi et al., 2000).

4.3.3. Effect of leptin on HPA axis

Levels of AVP mRNA in the PVN and the SON are increased by i.c.v. leptin (Yamamoto et al., 1999). I.c.v. leptin (100 pmol/rat) induces significant increases in the expression of CRH mRNA in the PVN and POMC mRNA in the pituitary and also increases plasma ACTH and corticosterone levels dose-dependently (peak occurred at 15 min) (Morimoto et al., 2000). This activation of the HPA axis was attenuated by i.c.v. pretreatment with a V1a or V1a/1b receptor antagonist but not with a V2 receptor antagonist. Hence, central leptin activates the HPA axis through AVP which in turn activates CRH neurones to drive ACTH and corticosterone (Morimoto et al., 2000).

4.3.4. Effect of leptin on magnocellular oxytocin and vasopressin neurones

Intracerebroventricular leptin treatment induces a time-dependent nuclear translocation of STAT3 immunoreactivity in hypothalamic nuclei, with strong nuclear STAT3 signals detectable in the ARC, LH and DMH, and less strong STAT3 immunoreactivity in the DHA, LHA, PHA, mPOA, vmPOA, PVN and SCN, but none in the SON (Hubschle et al., 2001). Central leptin administration increases AVP mRNA levels in the SON increases plasma AVP concentration in a dose-dependent manner (Yamamoto et al., 1999). The maximal effect is seen 15min after the administration of leptin (Yamamoto et al., 1999). However, i.c.v. leptin had no effects on plasma oxytocin secretion and gene expression in the SON (Yamamoto et al., 1999) and did not increase Fos expression in SON oxytocin neurones (Blevins et al., 2004). Central leptin administration increases the percentage of oxytocin neurones that show Fos activation in the pPVN, although the actual numbers are relatively small (Blevins et al., 2004). Interestingly, oxytocin receptor antagonist administered i.c.v. to 6h fasted rats 30-45min before i.c.v. leptin prevented leptin-induced attenuation of cumulative food intake (Blevins et al., 2004). Recently, it has been found that oxytocin and vasopressin mRNA expression were down-regulated during 24h fasting in mice and were restored by leptin administration (Tung et al., 2008).

Systemic leptin administration increases Fos expression in the pPVN, but not in oxytocin neurones (Elias et al., 2000). Systemic leptin did not induce pSTAT3 or Fos in the SON (Celine Caquineau, *personal communication*). However, systemic leptin treatment increases nuclear STAT5 in the SON (Mutze et al., 2007). STAT5 activation is seen only after a prolonged time interval (120-150min after leptin). Hence, leptin might induce the formation or release of a secondary mediator in the brain, which in turn, could account for nuclear STAT5 activation (Mutze et al., 2007). As SON neurones express leptin receptors (Hakansson et al., 1998) and systemic leptin activates vagal afferents (Wang et al., 1997) and brainstem neurones (Grill et al., 2002), it is possible that leptin either acts directly or indirectly on the SON neurones. As the activation of STAT5 does not indicate whether the neurones are excited or inhibited by leptin, electrophysiological studies become very important to determine the effects of leptin administration.

In an i*n vitro* study, bath application of leptin (10^{-8} – 10^{-12} M) induces mainly inhibitory response in SON neurones of Wistar rats, although a minority showed excitation (Honda et al., 2002). Tolbutamide (a K^+ channel blocker) did not reverse the inhibitory actions of leptin in the SON indicating that this effect is not by activation of ATP-sensitive K^+ channels (Honda et al., 2002). The inhibitory effect persisted with blockade of synaptic transmission by the perfusion of low Ca^{2+} and high Mg^{2+} medium, indicating that the

inhibitory effect of leptin on SON neurones is due to its direct action (Honda et al., 2002). However, the effect of leptin *in vivo* and the effect when leptin is administered via the systemic route on SON neurones, which is physiological, are not known.

4.3.5. Leptin action on the brainstem

Leptin receptor mRNA has been localized in rat brainstem (Elmquist et al., 1998; Mercer et al., 1998). Leptin receptor immunoreactivity colocalizes with TH (Tyrosine hydroxylase) and serotonin in the NTS and raphe nuclei (Hay-Schmidt et al., 2001). Systemic leptin induces Fos in the superior lateral and external lateral subdivisions of the parabrachial nucleus and the NTS of the brainstem (Elias et al., 2000), and STAT3 phosphorylation in the dorsal motor nucleus of the vagus nerve (DMNV), lateral parabrachial nucleus (PBel) and the NTS of the brainstem increases 30min after leptin administration (5 mg/kg; i.v.) (Hosoi et al., 2002b). These findings indicate that circulating leptin may directly act in the brainstem to elicit physiological responses, or that leptin administration might activate a neurotransmitter system that subsequently activates STAT3 signaling system in the brainstem (Hosoi et al., 2002b). On the contrary, although Ob-Rb leptin receptor expression was found in the AP region (Mercer et al., 1998), leptin-induced pSTAT3 immunoreactivity was not detected in that nucleus (Hosoi et al., 2002b). Hence the brain regions where Ob-Rb receptors exist do not always respond to physiologically circulating leptin (Hosoi et al., 2002a).

Leptin receptor is expressed in a variety of peripheral tissues (Tartaglia et al., 1995; Takaya et al., 1996). Leptin receptor is expressed in the vagas nerve (Buyse et al., 2001) and leptin has been shown to activate vagal afferent nerves (Wang et al., 1997; Yuan et al., 1999). Leptin-induced reduction in food intake is not abolished by vagotomy in rats and hence vagal afferents do not constitute a major route for mediating the regulatory effect of leptin on food intake, over a period of several hours (Sachot et al., 2007).

4.3.6. Leptin during fasting

Fasting reduces circulating leptin concentration (Maffei et al., 1995). With 3d of fasting in rats, leptin mRNA in fat fell dramatically; fasting levels of leptin mRNA were reduced to 12% of fed control levels (Frederich et al., 1995). Depriving male mice of food for 48h caused a 16% fall in weight and reduced circulating leptin from 3.87 ± 0.35 to 1.5 ± 0.11 ng/ml (Ahima et al., 1996). A 24h fast decreased plasma leptin significantly in both pregnant and non-pregnant rats compared with pre-fasting levels (Ladyman and Grattan, 2004). Leptin levels fall rapidly with the onset of starvation, disproportionate to changes in adipose tissue mass (Ahima et al., 1996). Fasting for 48h reduces SOCS3 mRNA in ARC and DMH compared to fed rats (Baskin et al., 2000). Fasting (for 3d) induced reduction in

circulating levels of leptin increases NPY and AgRP expression and decreases POMC and CART expression in the ARC, and decreases TRH mRNA in the PVN (Fekete et al., 2006). Central leptin administration reverses these changes (Fekete et al., 2006).

Starvation markedly activates the HPA axis (Chowers et al., 1969). Leptin administration in mice blunted the fasting-mediated surge in plasma ACTH and corticosterone (Ahima et al., 1996). In hypothalamic perifusion studies, hypoglycaemic stimulus-induced CRH release was inhibited by leptin in a dose-dependent manner (Heiman et al., 1997). Leptin does not directly alter pituitary release of ACTH (Heiman et al., 1997). Hence, this attenuation of the stress induced HPA axis response by leptin is exerted at the hypothalamic level to inhibit CRH release (Heiman et al., 1997).

4.3.7. Leptin resistance during pregnancy

4.3.7.1. Gestational profile of leptin

Pregnancy is a hypermetabolic state associated with a great increase in maternal body fat and weight. Positive energy balance is maintained during pregnancy by increase in food intake to prevent the depletion of maternal energy sources (Richard and Trayhurn, 1985). During pregnancy, despite the presence of elevated plasma leptin, food intake increases by up to 50% in rats (Cripps and Williams, 1975; Shirley, 1984; Ladyman and Grattan, 2004). Hence, pregnancy, a state of physiological hyperphagia, is an example of transient leptin resistance (Augustine et al., 2008).

Abdominal fat mass increases during pregnancy and is significantly higher than non-pregnant levels on day 14 to 21 of pregnancy (Ladyman and Grattan, 2004). Total abdominal fat mass on day 21 of pregnancy is almost double the amount in non-pregnant rats (Ladyman and Grattan, 2004). Leptin content in adipose tissues also increases during pregnancy compared with the non-pregnant state. It sharply declines to less than non-pregnant levels on day 21 (Kawai et al., 1997). An increase in leptin mRNA in fat was observed as pregnancy advances (Kawai et al., 1997; Garcia et al., 2000). Therefore, the rise in plasma leptin levels during pregnancy results not only from increase in fat mass but also from increased gene expression and protein production of leptin in adipose tissues (Kawai et al., 1997).

Whether the placenta is a source of leptin during pregnancy in rodents is controversial. It has been reported that placental (on day 12 and 19) and decidual (on day 12) tissues obtained from pregnant rats do not show any leptin mRNA expression (Kawai et al., 1997). Similar observations in placenta have been reported (Gavrilova et al., 1997). On the contrary, an increase in leptin mRNA level in rat placenta has been observed as pregnancy advances (Garcia et al., 2000). Also, placental leptin receptor expression, Ob-Rb in

particular, increases during pregnancy (Chien et al., 1997; Kawai et al., 1997; Szczepankiewicz et al., 2006).

4.3.7.2. Mechanisms of leptin resistance

Central leptin resistance may involve several mechanisms (Augustine et al., 2008) as described below.

4.3.7.2.1. Resistance – by receptor gene down regulation

Seeber *et al* reported that hypothalamic Ob-Rb mRNA expression is elevated in early pregnancy (Day 7) in rats but returned to pre-pregnancy levels by mid-gestation and remained thereafter (Seeber et al., 2002). Hence, changes in the absolute levels of Ob-Rb mRNA expression do not account for leptin resistance during pregnancy, but the increase in hypothalamic Ob-Rb in early pregnancy could potentially reset the sensitivity of the hypothalamus to leptin, such that the subsequent decline in Ob-Rb expression could contribute to leptin resistance (Seeber et al., 2002).

Hypothalamic Ob-Rb mRNA levels have been found to be significantly lower in pregnant (day 18) than in nonpregnant rats, but no changes were found in the content of the mRNAs encoding short forms of the leptin receptor (Ob-Ra, Ob-Rc, Ob-Re and Ob-Rf) (Garcia et al., 2000). These authors opined that this specific down regulation of the Ob-Rb at the hypothalamic level would explain, at least partially, the state of leptin resistance during pregnancy. Ob-Rb mRNA levels in the VMH decrease during pregnancy while they remained relatively constant in the ARC (Ladyman and Grattan, 2005). Thus, leptin resistance occurs at the hypothalamic level with a region-specific loss of hypothalamic Ob-R mRNA (Ladyman and Grattan, 2005).

4.3.7.2.2. Resistance – by reduced leptin transport across BBB

In contrast to the lack of change in pregnancy of the mRNA expression of short forms of the leptin receptors in the hypothalamus reported by Garcia *et al* (Garcia et al., 2000), Szczepankiewicz *et al* reported that both long and short forms of leptin receptor mRNA expression significantly decreased in the hypothalamus during pregnancy (Szczepankiewicz et al., 2006). The decrease in short forms of the receptors might mean that less amount of leptin cross the blood-brain barrier during pregnancy contributing to leptin resistance (Szczepankiewicz et al., 2006).

4.3.7.2.3. Resistance – by increase in plasma leptin binding activity

The increase in plasma leptin level during pregnancy could possibly be due to gradual and marked increase in plasma leptin-binding activity between dioestrus and late pregnancy (Seeber et al., 2002). The placenta produces large amounts of the OB-Re isoform of leptin receptor mRNA, which encodes a soluble binding protein (Gavrilova et al., 1997).

Plasma binding proteins are thought to restrict exit of leptin into extravascular tissues, thus reducing the metabolic clearance rate of leptin (Cumin et al., 1997a, b) and its access to target cells (Huang et al., 2001). Hence, the presence of leptin-binding proteins in the plasma during pregnancy may impair leptin action by restricting leptin access to the brain (Augustine et al., 2008).

4.3.7.2.4. Resistance – by alteration in leptin receptor signaling

In the ARC, NPY mRNA level is either unchanged (Rocha et al., 2003) or increased during pregnancy (Garcia et al., 2003) and levels of POMC mRNA also remain unchanged (Garcia et al., 2003; Rocha et al., 2003) or increased (Douglas et al., 2002). Similarly, ARC AgRP mRNA level is either unchanged (Ladyman, unpublished results; as cited in Augustine *et al.*, 2008) or increased (Rocha et al., 2003) during pregnancy. Either no change or elevated NPY and AgRP mRNA in the face of elevated leptin during pregnancy is further evidence for leptin resistance at the level of the ARC (Augustine et al., 2008). Though the overall level of pSTAT3 activation in the ARC after leptin administration in pregnant rats is reduced (Ladyman and Grattan, 2004), the number of leptin-responsive cells, measured by leptin-induced phosphorylation of STAT3 is not altered in the ARC in pregnant rats (Ladyman and Grattan, 2005). Hence, some signalling pathways other than those involving pSTAT3 might be altered during pregnancy (Augustine et al., 2008). Indeed, leptin action on NPY and AgRP neurones may be independent of STAT3 (Bates et al., 2003).

4.3.7.2.5. Resistance – induced by hormonal mechanisms

(1) By leptin

Chronic high leptin level has been shown to induce leptin resistance (Sahu, 2002), and prolonged infusions of high levels of leptin for more than 14d are required to induce resistance (Sahu, 2002; Zhang and Scarpace, 2006). However, nonpregnant ob/ob mice (genetically lacking leptin) can be treated with leptin for up to 6 months with no observed resistance to leptin, whereas pregnant ob/ob mice treated with leptin throughout pregnancy become leptin resistant at mid-pregnancy (Mounzih et al., 1998). In addition, levels of leptin rise slowly during pregnancy and are only elevated 2-3 fold for a few days prior to development of leptin resistance (Ladyman and Grattan, 2004). Hence, from these lines of evidence it is unlikely that leptin resistance during pregnancy is leptin-induced (Augustine et al., 2008).

(2) By 17β-oestradiol

17β-Oestradiol is anorectic as it reduces meal size (Eckel and Geary, 2001; Asarian and Geary, 2002) and increases energy utilization (Wade and Gray, 1979). It increases leptin

sensitivity in the brain (Clegg et al., 2006). Hence, low 17β-oestradiol levels during pregnancy might reduce leptin responsiveness (Augustine et al., 2008).

(3) By progesterone

Administration of progesterone and its metabolites increases food intake in rats (Hervey and Hervey, 1967; Wade and Gray, 1979; Chen et al., 1996). Progesterone treated rats show increased food intake and body weight but maintain normal plasma and CSF leptin levels (Grueso et al., 2001), which indicates that progesterone might inhibit CNS actions of leptin (Augustine et al., 2008).

(4) By prolactin and placental lactogen

Prolactin receptors are expressed in the hypothalamic regions containing orexigenic neurones (Augustine et al., 2003; Kokay and Grattan, 2005). Prolactin infusion induces hyperphagia in a dose-dependent manner in virgin rats (Gerardo-Gettens et al., 1989; Sauve and Woodside, 1996). This was found to be a central effect with direct actions of prolactin in the hypothalamus (Sauve and Woodside, 1996). During pregnancy, levels of prolactin and its closely related hormone, placental lactogen, are elevated (Grattan, 2001), which might contribute to pregnancy induced hyperphagia (Augustine et al., 2008). Leptin resistance develops between day 7 and 14 of pregnancy coincident with the development of the placenta and the onset of secretion of placental lactogen (Ladyman and Grattan, 2004). Early pregnant and pseudopregnant rats show pulsatile prolactin secretion and hyperphagia, but continue to have a relatively small response to leptin (Ladyman and Grattan, 2004; Augustine and Grattan, 2008). In contrast, during the second half of pregnancy, when placental lactogen production is chronically elevated, animals show a loss of response to leptin (Ladyman and Grattan, 2004). Augustine and Grattan (2007) induced leptin resistance in pseudopregnant rats by giving chronic i.c.v. prolactin infusions to mimic the pattern of placental lactogen secretion during the second half of pregnancy. Hence, chronically high lactogen levels, secreted by the placenta during the second half of pregnancy may be responsible for central leptin resistance and hyperphagia possibly through DMH NPY neurones that possess prolactin receptors (Ladyman and Grattan, 2004; Augustine and Grattan, 2008).

4.4. Methods

4.4.1. Experiment: Effect of systemic administration of leptin on the electrical activity of SON oxytocin and vasopressin neurones in urethane-anaesthetised fasted/unfasted virgin/pregnant rats.

Urethane-anaesthetized virgin and pregnant (19-20d of pregnancy) rats were fixed with an i.v. cannula on the day of the experiment. The SON and neural stalk were exposed via the ventral transpharyngeal surgery. After identifying SON neurones, oxytocin neurones were differentiated from vasopressin neurones. After an initial basal recording of firing rate in SON oxytocin neurones for 10min, leptin was administered i.v. in 100µl normal saline and the response was recorded. Both unfasted virgin and pregnant groups were treated with a same dose of leptin (100µg) irrespective of the body weight. Fasted virgin and pregnant rats were treated with 1µg, 10µg, 100µg or 1mg leptin to study the dose response. Vasopressin neurones were studied only with 100µg dose of leptin. The groups studied are:

- For oxytocin neurones:
  - Unfasted Virgin: n = 23 (100µg)
  - Unfasted Pregnant: n = 5 (100µg)
  - Fasted Virgin:
    - 1µg leptin: n = 6
    - 10µg leptin: n = 6
    - 100µg leptin: n = 8
  - Fasted Pregnant:
    - 1µg leptin: n = 3
    - 10µg leptin: n = 6
    - 100µg leptin: n = 8
    - 1mg leptin: n = 6
- For vasopressin neurones:
  - Phasic: n = 8 (100µg)
  - Non-phasic: n = 13 (100µg)

4.5. Results

4.5.1. Body weight

The pregnant rats had significantly higher body weight than the virgin rats whether they were fasted or unfasted while fasting did not alter the body weight significantly both in virgin and pregnant rats (Fig. 4.2).

4.5.2. SON oxytocin neurone basal firing rates

The basal firing rate of SON oxytocin neurones in unfasted pregnant rats was significantly higher than that of unfasted virgin rats (Fig. 4.3). The basal rates did not differ significantly between other groups.

4.5.3. Effect of systemic leptin administration on electrical activity of SON oxytocin neurones in fasted/unfasted virgin and pregnant rats

4.5.3.1. Unfasted virgin rats (n=23):

Leptin administration (100µg; i.v) excited 16/23 SON oxytocin neurones (e.g. Fig. 4.4), inhibited 4/23 neurones and had no effect on 3/23 neurones. On average, the basal rate of 3.4 ± 0.4 spikes/s was increased by 0.4 ± 0.08 spikes/s 1.5min after leptin injection (n=23, Fig. 4.5). The rate returned to basal firing rate by 10min. The mean firing rate during 0-10min after leptin was significantly higher than during the basal period (P=0.01, paired t-test). The incidence of a spike occurring within 0-0.5s after a spike was significantly increased 0-10min after leptin compared to that during the basal 10min period (P=0.004, paired t-test, Fig. 4.6a). The descending slopes of the histograms fit well with exponential curves which is a characteristic of oxytocin neurones (Fig. 4.6b). There was no change in the shape of the hazard plot (Fig. 4.7) or in the ratio of peak early (<0.07s) to mean late (0.2-0.3s) hazard before and after leptin injection (Fig. 4.8).

4.5.3.2. Unfasted virgin rats (n=23) *vs.* unfasted pregnant rats (n=5):

Leptin excited all five SON oxytocin neurones tested in unfasted pregnant rats (e.g. Fig. 4.9); this compares with excitation of 16/23 SON oxytocin neurones in unfasted virgin rats. The basal rate of 3.4 ± 0.4 spikes/s was increased by 0.4 ± 0.08 spikes/s at 1.5min after leptin in unfasted virgin rats while the basal rate of 6.65 ± 0.4 spikes/s was increased by 0.8 ± 0.2 spikes/s at 17min after leptin in unfasted pregnant rats. The rates returned to basal by 10min in unfasted virgin rats and by 30min in unfasted pregnant rats. Overall, the effect of leptin was greater and more prolonged in unfasted late pregnant rats than in unfasted virgin rats (Fig. 4.10).

4.5.3.3. Fasted virgin rats (n=8) *vs.* fasted pregnant rats (n=8):

In fasted virgin rats, 5/8 SON oxytocin neurones were excited, 2/8 were inhibited and 1/8 did not respond. In fasted pregnant rats, 2/8 neurones were excited, 2/8 were

inhibited and 4/8 did not respond. In fasted virgin rats, overall, the basal rate of 4.45 ± 0.75 spikes/s was increased by 1 ± 0.5 spikes/s, on average, 25.5min after leptin and it returned to basal by 1h (Fig. 4.11a and 4.11b). This apparent increase (including cells that were excited, inhibited or not affected) during the entire period of observation after leptin injection was not statistically significant compared to the average basal rate. However, the excitation observed 0-10min after leptin was nearly significant (P=0.051, paired t-test; Fig. 4.11a and 4.11b).

In fasted pregnant rats, the basal rate was 4.6 ± 0.65 spikes/s and, on average, there was no change in the firing rate after leptin treatment compared to the basal rate. The response after leptin did not differ significantly between fasted virgin and pregnant rats (Fig. 4.11a and 4.11b). Hence, the excitation of SON oxytocin neurones by i.v. leptin in fasted virgins was evidently increased by fasting (see below and Fig. 4.12a and 4.12b). In contrast, the excitatory response of SON oxytocin neurones to i.v. leptin in late pregnant rats was reduced by fasting, and essentially suppressed (see below and Fig. 4.13).

4.5.3.4. Unfasted virgin rats (n=23) *vs.* fasted virgin rats (n=8):

In unfasted virgin rats 70% of SON oxytocin neurones were excited (16/23 excited, 4/23 inhibited and 3/23 did not respond) and in fasted virgin rats 62.5% of oxytocin neurones were excited (5/8 excited, 2/8 inhibited and 1/8 did not respond). The basal rate of 3.4 ± 0.4 spikes/s was increased by 0.4 ± 0.08 spikes/s by 1.5min after leptin in unfasted virgin rats while the basal rate of 4.45 ± 0.75 spikes/s was increased by 1 ± 0.5 spikes/s 25.5min after leptin in fasted virgin rats (Fig. 4.12a, b). The rates returned to basal firing rate by 10min and 1h in unfasted and fasted virgin rats, respectively. In unfasted virgin rats, the mean change in firing rate 0-10min after leptin was significantly higher than basal (P=0.01, paired t-test) while in fasted virgin rats, the excitation was nearly significant (basal *vs.* 0-10min after leptin: P=0.051; basal *vs.* 20-30min after leptin: P=0.2; paired t-test). The response after leptin did not differ significantly between the two groups (Fig. 4.12a, b).

4.5.3.5. Unfasted pregnant rats (n=5) *vs.* fasted pregnant rats (n=8):

All the SON oxytocin neurones were excited by leptin in unfasted pregnant rats (n=5) while overall there was no response in fasted pregnant rats (2/8 excited, 2/8 inhibited and 4/8 did not respond). In unfasted pregnant rats, the basal rate of 6.65 ± 0.4 spikes/s was increased by 0.8 ± 0.2 spikes/s 17min after leptin and it returned to basal by 30min. The excitatory response after leptin was significantly higher than the basal firing rate (P=0.006, paired t-test). The basal firing rate in fasted pregnant rats was 4.6 ± 0.65 spikes/s and there was no response, on average, to leptin injection. However, the changes in firing rate after leptin did not differ significantly between these groups (Fig. 4.13).

4.5.4. Dose dependent effects of leptin on electrical activity of SON oxytocin neurones in fasted virgin and pregnant rats

4.5.4.1. Fasted virgin rats:

As the dose of leptin was increased, the number of SON oxytocin neurones excited increased: 2/6 neurones were excited when 1µg leptin was injected (4/6 did not respond), 3/6 neurones were excited when 10µg leptin was injected (3/6 were inhibited) and 5/8 neurones were excited when 100µg leptin was injected (2/8 inhibited and 1/8 did not respond). The leptin-induced change in firing rate was not significantly different from the basal rate in the 1µg and 10µg leptin groups ($P>0.05$) while in the 100µg group, there was a mild excitatory response after leptin which was nearly significant ($P=0.051$, paired t-test). However, the mean change in firing rate 0-10 and 0-20min after leptin was not significantly different between the groups (Fig. 4.14).

4.5.4.2. Fasted pregnant rats:

Overall, there was no response of SON oxytocin neurones in fasted pregnant rats to leptin injected at the various doses. None of the three neurones tested responded to 1µg leptin; a few were excited after 10µg (2/6 excited, 3/6 inhibited and 1/6 did not respond), 100µg (2/8 excited, 2/8 inhibited and 4/8 did not respond) and 1mg (2/6 excited, 2/6 inhibited and 2/6 did not respond) leptin. On average, there was no significant change in firing rate within and between different dose groups after administration of leptin (Fig. 4.15).

Comparing the dose response in fasted virgin/pregnant rats, there was no influence of physiological status (virgin/pregnant) on the response to different doses of leptin (1/10/100µg) administered ($F_{2, 29}= 0.5$, $P=0.6$, two-way ANOVA). The responses of oxytocin neurones were not different between virgin and pregnant rats ($F_{1, 29}=2.7$, $P=0.1$, two-way ANOVA) or between different doses of leptin ($F_{2, 29}=0.17$, $P=0.8$, two-way ANOVA; Fig. 4.16a, b and 4.11b).

4.5.5. Effect of systemic administration of leptin on electrical activity of SON vasopressin neurones in unfasted virgin rats

4.5.5.1. Non-phasic vasopressin neurones (n=13)

The response to leptin (100µg; i.v) was predominantly inhibitory in non-phasic vasopressin neurones [6/13 inhibited (e.g. Fig. 4.17), 4/13 excited and 3/13 did not respond]. On average, the basal rate of 8.4 ± 1.2 spikes/s was decreased by 0.6 ± 0.45 spikes/s 1min after leptin (n=13). The rate returned to basal level by 2min. This transient inhibitory response was not significantly different from the basal (Fig. 4.18).

4.5.5.2. Phasic vasopressin neurones (n=8)

Overall, phasic vasopressin neurones were little affected by leptin, however, a few neurones were inhibited (e.g. Fig. 4.19). During 0-5min after leptin administration the activity quotient decreased in 6/8 neurones and increased in 2/8 neurones, frequency within bursts decreased in 4/8 neurones and increased in 4/8 neurones and mean interburst interval increased in 4/8 neurones and decreased in 4/8 neurones. Overall, activity quotient, frequency within bursts and mean interburst interval of phasic vasopressin neurones were not altered by leptin (Fig. 4.20, 4.21 and 4.22).

4.5.5.3. Interspike interval histogram and hazard (n=19)

There was no change in the interspike interval histogram after leptin administration (Fig. 4.23a). The distal tail of the histograms fitted well with the exponential curves while the proximal slope of the histogram of less than 0.2s intervals were left unfit, characterising vasopressin neurones (Fig. 4.23b). There was no change in the shape of the hazard plot after leptin administration (Fig. 24).

4.6. Discussion

4.6.1. Leptin-induced excitation of SON oxytocin neurones

Systemic leptin administration resulted in a significant excitatory effect on SON oxytocin neurones in unfasted virgin rats. Direct action of leptin on SON neurones results in inhibition of electrical activity *in vitro* (Honda et al., 2002) and i.c.v. leptin has no effect on oxytocin mRNA expression in the SON or on plasma oxytocin level (Yamamoto et al., 1999). Hence, the excitatory effects observed in this study might involve indirect actions of leptin.

4.6.2. Rapid effects of leptin

Leptin is a long term satiety signal. Leptin-induced reduction in food intake had a delayed onset (4-5h) and lower potency when injected systemically compared with central administration (30min onset) (Cusin et al., 1996; Barrachina et al., 1997; Thiele et al., 1997). However, it is interesting that the leptin induced excitation of SON oxytocin neurones observed in this study were rapid. The peak excitation was seen within 2min after injection and the effect lasted only for 10min. Systemic leptin activates STAT5 in SON neurones 120min after injection (Mutze et al., 2007). However, systemic leptin activates STAT3 in mouse hypothalamus within 15min and it shows a peak response at 30min (Vaisse et al., 1996). I.c.v. leptin increases plasma corticosterone with the peak occurring at 15 min (Morimoto et al., 2000). Leptin inhibits both release (Stephens et al., 1995) and synthesis of NPY (Stephens et al., 1995; Schwartz et al., 1996). Similar rapid responses to leptin have also been observed in other studies (Spanswick et al., 1997; Powis et al., 1998; Honda et al.,

2002). The change in neuronal responses observed in some studies is too rapid to be accounted for by genomic mechanisms (Honda et al., 2002). The acute regulation of orexigenic ARC neurones by leptin does not require STAT3 mediated transcription (Munzberg et al., 2007), but it is likely to be the result of inhibition of excitatory postsynaptic membrane potentials in NPY neurones (Glaum et al., 1996). Leptin inhibits hippocampal neurones by increasing K^+ conductance (Shanley et al., 2002a; Shanley et al., 2002b). Similarly, as the electrical activation of SON oxytocin neurones observed in the present study was too rapid to be accounted for by leptin receptor signalling involving STAT3 or STAT5 or any other signalling mechanisms involving gene transcription, these rapid actions of leptin could be possibly through non-genomic mechanisms either directly involving SON neurones or indirectly via modification of excitatory inputs to SON neurones.

Indirect action of systemically administered leptin on SON neurones could be possibly via ARC, VMH and/or brainstem neurones as these nuclei express leptin receptors (Hakansson et al., 1998; Grill et al., 2002) and are direct targets for leptin (Elias et al., 2000). In addition, ARC and NTS neurones project to the SON (Alonso and Assenmacher, 1984; Leng et al., 1988). Indirect actions of leptin on the SON could also be via activation of the vagal afferents that express leptin receptors (Buyse et al., 2001; Burdyga et al., 2002) which in turn activates brainstem neurones resulting in activation of SON oxytocin neurones.

4.6.3. Leptin-induced excitation of SON oxytocin neurones: Physiological implications

Natriuretic peptides such as atrial natriuretic peptide (ANP) regulate postprandial natriuresis to establish normal plasma sodium and volume after food intake (McCann et al., 2003). Oxytocin is released postprandially and it mediates the release of ANP (Haanwinckel et al., 1995). The natriuretic effect of peripheral oxytocin is also via direct binding of oxytocin to its receptors on NOergic cells in the macula densa and renal proximal tubules to release cGMP that closes Na^+ channels, and also via generation of NO leading to increased cGMP (Soares et al., 1999).

Similar to oxytocin, leptin also is an important renal sodium-regulating factor under conditions of mild sodium and volume expansion (Villarreal et al., 2006). Leptin receptors are expressed in the kidney and systemic administration of leptin induces a significant and rapid diuretic and natriuretic effect in conscious and anaesthetized rats (Jackson and Li, 1997; Serradeil-Le Gal et al., 1997; Beltowski et al., 2004). Leptin-induced natriuresis is through direct binding of leptin to its receptors in renal tubules and is mediated by NO (Villarreal et al., 2004). Leptin antibody significantly decreases urinary sodium excretion and urinary flow by 30% compared to control rats (Villarreal et al., 2006). It is possible that, physiologically, in addition to its direct effects on renal tubules, leptin might act indirectly

through oxytocin promoting natriuresis. Transient increase in leptin mRNA expression seen in adipose tissue after food intake (Saladin et al., 1995) supports the possibility that postprandial natriuresis could be partly mediated by leptin through oxytocin.

The increase in firing rate of SON oxytocin neurones in response to leptin observed in this study might accompany dendritic release of oxytocin through which leptin, at least in part, might mediate its anorectic effects. This is supported by the report that leptin-induced reduction in food intake was reduced by central administration of oxytocin receptor antagonist (Blevins et al., 2004).

4.6.4. Leptin-induced excitation of SON oxytocin neurones in pregnant rats

As pregnancy is a physiological state of leptin resistance (Augustine et al., 2008) and oxytocin neurone responses to various stimuli are attenuated during pregnancy (Douglas et al., 2007), leptin-induced excitation of SON oxytocin neurones observed in virgin rats was expected to be absent or less in late pregnant rats. Surprisingly, however, in unfasted pregnant rats, leptin had similar excitatory, but more prolonged effects on SON oxytocin neurones than in virgin rats, indicating that these neurones are not resistant to systemic leptin during pregnancy.

Leptin resistance occurs at the hypothalamic level with a region-specific loss of hypothalamic Ob-R mRNA (Ladyman and Grattan, 2005). It is not known whether Ob-R expression in the SON is altered during pregnancy, a lack of such alteration might be responsible for this persistent excitatory response observed in pregnant rats. As leptin excites oxytocin neurones both during the non-pregnant and pregnant state and Ob-Rb mRNA levels remain relatively constant in the ARC in pregnancy (Ladyman and Grattan, 2005), it is possible that systemic leptin acts on SON neurones via the ARC.

As the pregnant rats were given the same dose as virgin rats, dose per 100 gram BW would have been less as the body weight is significantly increased during late-pregnancy. Pregnant rats with higher body weight have higher circulating leptin levels, but also higher circulating leptin binding proteins (Augustine et al., 2008). Hence, the actual effective concentration of leptin in the pregnant rats was unknown. However, the oxytocin neurones in pregnant rats responded similarly to the dose as virgin rats. Though these responses in virgin and pregnant rats may not be directly comparable due to the discrepancy in the dose per gram BW given and due to the physiological changes during pregnancy altering the effective circulating concentration of leptin, this study showed that oxytocin neurones are not resistant to leptin during pregnancy.

4.6.5. Leptin-induced excitation of SON oxytocin neurones in pregnant rats: Physiological implications

Total circulating blood volume and sodium are increased during pregnancy (Brunton et al., 2008). Oxytocin neurone responses are contained in pregnancy, which should maintain this altered physiological status so as to enable sufficient blood supply to the gravid uterus (Brunton et al., 2008). Hence, it is surprising that acute leptin activates SON oxytocin neurones and hence presumably increases oxytocin secretion even during pregnancy. As leptin receptor signalling is similar to signalling by other cytokines, an acute bolus injection of leptin might be perceived as a strong cytokine signal to activate SON oxytocin neurones in pregnant rats, which otherwise are under control by several inhibitory mechanisms during pregnancy (Brunton et al., 2008).

4.6.6. Effect of fasting on leptin-induced excitation of SON oxytocin neurones: Lack of dose-dependent response

Fasting was expected to either increase (due to increase in the sensitivity to leptin after reduction in the circulating leptin level by fasting) or decrease (to avoid natriuresis so as to conserve sodium and water during fasting) leptin-induced excitation of SON oxytocin neurones. The results show that fasting did not potentiate the acute effects of leptin in virgin and pregnant rats. In addition, though leptin-induced change in firing rate was not different from basal rate both in fasted virgin and pregnant rats and though this reduced response was not significantly lesser than the unfasted virgin and pregnant rats, fasting seems to have attenuated the response in pregnant rats as they did not respond to leptin. Lack of food intake also reduces water intake in rats, but does not alter the plasma osmolality (Bruno et al., 1985). However, reduced circulating sodium level and plasma volume might attenuate the excitatory responses of oxytocin neurones to leptin so as to preserve the available sodium by avoiding oxytocin-induced natriuresis.

The response to 100µg leptin was almost significant (P=0.051) in fasted virgin rats compared to basal but there was no response to lesser doses. The fasted pregnant rats did not respond to any of the doses, even to the maximal 1mg dose. Fasting-induced homeostatic mechanisms might tightly regulate oxytocin neurone responses to preserve sodium level. On the other hand, oxytocin neurones in fasted rats might dose-dependently respond to leptin had the doses been higher. In addition, as this model of bolus injection of leptin does not resemble the slow physiological release, perhaps leptin did not reveal its physiological effect.

4.6.7. Leptin perceived as inflammatory stimuli in unfasted animals and compensates for reduced levels in fasted animals?

Leptin levels are acutely increased by inflammatory stimuli such as LPS and hence leptin induction is part of the acute phase response to inflammation (Grunfeld et al., 1996; Sarraf et al., 1997; Faggioni et al., 1998). The acute bolus injection of leptin might be perceived as an inflammatory stimulus exciting the oxytocin neurones as oxytocin is a stress hormone in the rat (Lang et al., 1983). During fasting, acute injection of leptin might just compensate for the reduced circulating levels and might not be perceived as an inflammatory stimulus and hence the oxytocin neurone responses are not altered in fasted rats. However, this concept does not explain why leptin excites oxytocin neurones in unfasted pregnant rats in which oxytocin neurone responses to inflammatory stimuli such as IL-1β are suppressed during pregnancy (Brunton et al., 2006).

4.6.8. Leptin-induced oxytocin secretion: trigger for parturition?

Withdrawal of leptin from pregnant ob/ob mice results in delay in parturition, with a prolongation of gestation for over 2 days (Mounzih et al., 1998) suggesting that an adequately higher circulating level of leptin is mandatory for a normal gestation period. This study found that fasting, which results in reduced leptin level and reduced availability of energy sources, attenuated leptin-induced excitation of oxytocin neurones and thus oxytocin secretion in fasted pregnant rats. This may have the effect of delaying parturition, whereas an acute increase in leptin level, above the high leptin level in unfasted pregnant rats, might signal availability of surplus energy sources, and hence trigger parturition through oxytocin secretion.

4.6.9. Leptin-induced effects on the electrical activity of SON vasopressin neurones

Systemic leptin either inhibited or did not affect SON vasopressin neurone activity in this *in vivo* study. As direct action of leptin on SON neurones results in inhibition of electrical activity *in vitro* (Honda et al., 2002), leptin-induced effects on vasopressin neurones observed in this *in vivo* study could be due to direct/indirect effects of leptin on SON vasopressin neurones.

The effect of systemic leptin on SON oxytocin neurones (excitatory) and vasopressin neurones (inhibitory or no effect) is similar to that of systemically administered CCK. Hence, it is possible that leptin-induced effects on SON neurones are mediated through CCK possibly involving abdominal vagal afferents and brainstem noradrenergic neurones. An interaction between CCK and leptin at the level of gastric vagal afferents (Yuan et al., 2000) support this hypothesis.

4.7. Conclusion

Systemic administration of leptin predominantly increases the electrical activity of SON oxytocin neurones and either inhibits or does not affect vasopressin neurones. SON oxytocin neurones are not resistant to leptin during pregnancy. Fasting did not alter leptin-induced excitatory responses in SON oxytocin neurones in virgin and pregnant rats. Physiologically, leptin may regulate appetite and electrolyte balance through SON oxytocin neurones.

CHAPTER V

SECRETIN

5.1. Introduction

Secretin is a twenty seven amino acid peptide from the S-cells of the duodenum. It is released in response to acidic contents entering the duodenum from the stomach (Bayliss and Starling, 1902). Suppression of gastric acid secretion with a H_2 blocker completely arrests release of secretin after a meal in dogs (Kim *et al.*, 1979). Pancreatic exocrine secretion after a meal is mainly stimulated by synergistic interaction between secretin and cholecystokinin (Chey *et al.*, 1984).

Peripheral injections of secretin result in Fos expression in the brain including the hypothalamus and brainstem (Goulet *et al.*, 2003; Yang *et al.*, 2004b). Intravenous (i.v) injection of secretin (40µg/kg) increases Fos expression in the supraoptic nucleus (SON) significantly (Yang *et al.*, 2004b). Intracerebroventricular (i.c.v) administration of secretin (1µg) increased Fos expression in SON oxytocin and vasopressin neurones and also increased peripheral release of oxytocin and vasopressin dose dependently (0.1 - 10µg; Takayanaki and Onaka, 2007). Bath application of secretin (2-50µM) increased the release of oxytocin and vasopressin from hypothalamic explants (Takayanaki and Onaka, 2007). Secretin and its receptor are found in the SON and the magnocellular area of the paraventricular nucleus (mPVN) (Chu et al., 2006a), and these hypothalamic regions also contain secretinergic neurones (Welch *et al.*, 2004). Secretin is also released from hypothalamic explants (Chu et al., 2006a). Hence, secretin is considered as a neuropeptide though its physiological role in the CNS is not established yet (Ng *et al.*, 2002).

As the gut is the primary source of secretin and as the role of peripheral secretin is well established, systemic administration of secretin for experiments on central actions seem more physiological than central administration. Though Fos studies show that SON neurones are activated by systemic administration of secretin, they do not allow 'real-time' measurement of neuronal activity. In addition, as there are no double immunohistochemical studies of Fos and oxytocin/vasopressin available, whether SON oxytocin or vasopressin neurones are activated by peripheral secretin injection is not known. Moreover, the duration and intensity of activation of individual oxytocin or vasopressin neurones in response to systemic secretin administration is not known. Dose dependent (0.4 – 40 µg/kg; i.v) increases in the number of cells expressing Fos in the CeA and AP have been reported (Goulet *et al.*, 2003). Similarly, dose dependent (40 and 100µg/kg; i.p) increases in the number of Fos expressing cells in the CeA have been reported (Yang *et al.*, 2004b). However, it is not known whether the neuronal activity of individual cells is dose dependent.

Hence the present study was aimed at studying the *in vivo* electrophysiological responses of SON oxytocin neurones to intravenous administration of secretin. The plasma oxytocin release following systemic secretin was also studied. In addition, as there is no evidence that secretin decreases food intake in rats, systemic secretin-induced somato-dendritic oxytocin release from the SON was also studied, with the hypothesis that secretin does not induce dendritic release of oxytocin.

Central administration of secretin increased the Fos expression in SON vasopressin neurones and peripheral release of vasopressin (Takayanagi and Onaka, 2007). Bath application induced vasopressin release from hypothalamic explants (Takayanagi and Onaka, 2007). However, the response of SON vasopressin neurones to systemic administration of secretin was not known. Hence, experiments were aimed to study whether secretin-induced responses in SON vasopressin neurones are similar to those induced by CCK, a functionally similar peptide (Chey and Chang, 2001), which when administered intravenously, either inhibits or does not affect the firing rate of SON vasopressin neurones (Verbalis et al., 1986a; Verbalis et al., 1986b; Renaud et al., 1987).

Electrophysiological studies on nodose ganglia in the rat suggest that secretin activates vagal primary afferent neurones (Li *et al.*, 2005). Secretin induces Fos expression in the NTS, AP and LC regions of the brainstem (Yang et al., 2004b). Moreover, *in vitro* studies found that secretin depolarizes neurones in the nucleus tractus solitarius (NTS) through activation of a nonselective cationic conductance (Yang et al., 2004a). The SON receives noradrenergic excitatory inputs from the NTS (Onaka et al., 1995a). In addition, α-1 adrenergic receptor activation induces release of oxytocin and vasopressin from hypothalamic explants (Randle *et al.*, 1986). To investigate the possible involvement of excitatory adrenergic pathways in mediating secretin-induced responses in SON neurones, the effect on responses to secretin of administering benoxathian hydrochloride (a competitive α-1 adrenergic antagonist) by microdialysis onto the SON or by i.c.v. infusion was also studied.

Oxytocin release during feeding might prevent the disturbances in water and electrolyte homeostasis which would otherwise occur as a result of food intake (Johnstone *et al.*, 2006). However, in the unfed state, the activation of SON oxytocin neurones might be suppressed to avoid natriuresis and diuresis. Hence, the basal activity of SON neurones was expected to be less in fasted rats. However, though the entry of acidic contents from the stomach to duodenum is the signal for physiological release of secretin (Bayliss and Starling, 1902), fasting also increases plasma secretin concentration in humans and dogs (Mason *et al.*, 1979; Manabe *et al.*, 1987). If it is also true in rats, the fasting-induced increase in the

circulating levels of secretin might actually increase the basal rate of oxytocin neurones. In such a case, exogenous secretin injection might not excite SON oxytocin neurones. To test these hypotheses, the effect of fasting on basal activity and secretin-induced increase in the activity of the SON neurones was also studied.

5.2. Hypotheses

1. Systemically administered secretin, at physiological doses, increases the electrical activity of SON oxytocin neurones and either inhibits or does not affect vasopressin neurones, similar to CCK.
2. Secretin dose-dependently excites SON oxytocin neurones and increases peripheral release of oxytocin in urethane anaesthetized female rats.
3. Systemic secretin does not induce somato-dendritic oxytocin release from the SON.
4. Similar to CCK, central noradrenergic pathways are involved in the secretin-induced excitation of SON oxytocin neurones.
5. Basal activity and secretin-induced excitation of SON oxytocin neurones are attenuated in fasted state.

5.3. Objectives

1. To study the electrophysiological responses of SON oxytocin and vasopressin neurones to intravenous administration of secretin in urethane anaesthetized female rats *in vivo.*
2. To evaluate whether secretin-induced effects on SON oxytocin neurones and plasma oxytocin concentration in urethane-anaesthetized female rats are dose dependent.
3. To study the systemic secretin-induced somato-dendritic oxytocin release from the SON.
4. To study the involvement of adrenergic pathways in secretin-induced effects on SON oxytocin neurones in urethane anaesthetized female rats.
5. To study the basal activity and secretin-induced change in the activity of SON oxytocin neurones in fasted rats.

5.4. Background

5.4.1. Secretin and its peripheral actions

Secretin was discovered more than 100 years ago (Bayliss and Starling, 1902). It was the first hormone to be discovered and the concept of a 'hormone' began from its discovery. It belongs to the family of brain-gut peptides: the secretin/glucagon/VIP superfamily that includes pituitary adenyl cyclase activating peptide (PACAP), growth hormone releasing hormone (GHRH), peptide histidine isoleucine (PHI), peptide histidine methionine (PHM), glucagon-like peptide 1 and 2 (GLP-1 and GLP-2) and gastric inhibitory polypeptide (GIP) (Sherwood *et al.*, 2000).

Secretin functions via a cell surface receptor, the secretin receptor. The secretin receptor and the receptors for the secretin/glucagon/VIP superfamily are grouped under the B1 subclass in the G protein-coupled receptor (GPCR) superfamily (Siu *et al.*, 2006). Secretin plays a key role in the physiology of the gastro-intestinal tract via activation of its receptors in nanomolar concentrations.

Postprandially released secretin is transported to the pancreas and liver. Activation of secretin receptors leads to the elevation of cAMP or Ca^{2+} signalling pathways (Trimble *et al.*, 1987). Stimulation of these pathways activates various ion transporters in the pancreatic and biliary duct epithelial cells, resulting in exocrine secretion of bicarbonate ions, water and other electrolytes to neutralise acidic gut contents so that an optimum pH is available for intestinal enzymes to act on food (Ishiguro *et al.*, 2002).

Secretin receptors are expressed in the cholangiocytes in the liver (Alpini et al., 1994). Secretin, via cAMP and PKA pathway, increases bicarbonate rich bile secretion from the liver (Kanno et al., 2001). Secretin also acts on the proximal and distal epididymis to stimulate bicarbonate rich epididymal secretion in the rats (Chow et al., 2004).

Secretin receptors are present on vagal afferent fibres (Wang et al., 1995). The physiological action of secretin is highly dependent on the vagal afferent pathway. Thus, chemical ablation of vagal afferent fibres by perivagal application of capsaicin in rats resulted in a profound inhibition of the pancreatic secretion stimulated by a physiological, but not by a pharmacological, dose of secretin (Li *et al.*, 1995).

Physiological functions of secretin also include inhibition of gastric acid secretion, gastrin release and gastric emptying (Wormsley and Grossman, 1964; Vagne and Andre, 1971; Straus et al., 1975) through a vagal-dependent pathway (Raybould and Holzer, 1993; Lu and Owyang, 1995; Li et al., 1998). Supraphysiologic blood levels of secretin (100-fold endogenous levels) affect pancreatic fluid release by a mechanism independent of the vagus (Lu and Owyang, 1995).

The half life of circulating secretin is 4.1min in humans (Kolts and McGuigan, 1977) and 1.5 - 3min in rats (de Smul et al., 1974; Lehnert et al., 1974; Tanaka and Katayama, 1984). The release of secretin is controlled by a feedback mechanism mediated by pancreatic proteases (Green and Lyman, 1972). Secretin has been used for many years for evaluating the function of the exocrine pancreas (Dreiling and Hollander, 1948).

5.4.2. Secretin and its actions in the brain

^{125}I-[Tyr10]-Secretin was found to cross the blood–brain barrier and entered every brain region, with fastest uptake found in the hypothalamus and hippocampus (Banks *et al.*, 2002). Secretin crosses the vascular barrier by a nonsaturable process and the choroid plexus by a saturable process (Banks *et al.*, 2002). Secretin promotes adenylate cyclase activity (Karelson *et al.*, 1995), stimulates cAMP accumulation (Karelson *et al.*, 1995) and modulates catecholamine metabolism (Fuxe et al., 1979; Ip and Zigmond, 2000) in the brain. It also increases TH activity in the hypothalamus (Babu and Vijayan, 1983), and increases glutamate and GABA levels in rat hippocampus (Yung *et al.*, 2001; Kuntz *et al.*, 2004). Central (i.c.v) administration of secretin stimulates pancreatic exocrine secretion in rats (Conter *et al.*, 1996). Indeed, it is more potent in stimulating pancreatic secretion when given into the brain than when given intravenously (Conter *et al.*, 1996).

Secretin (O'Donohue *et al.*, 1981; Yung *et al.*, 2001), secretin mRNA (Itoh *et al.*, 1991; Ohta *et al.*, 1992; Yung *et al.*, 2001), secretin receptor (Fremeau *et al.*, 1983; Nozaki *et al.*, 2002) and receptor mRNA (Yung *et al.*, 2001) have been detected in the brain. However, Kopin *et al* failed to detect secretin mRNA in the central nervous system (CNS) (Kopin *et al.*, 1990). Secretin is also found in the brainstem (Koves *et al.*, 2002), and secretinergic neurones are found in the mPVN and SON (Welch *et al.*, 2004). However, Koves *et al* and Ng *et al* failed to find secretin in the hypothalamus (Koves *et al.*, 2002; Ng *et al.*, 2002). Recent studies have indicated that secretin indeed may function as a neuropeptide. For example, secretin specifically stimulated adenylate cyclase in hypothalamus and hippocampus (Karelson *et al.*, 1995). Hence, secretin seems to act as a peripheral hormone and central neuropeptide with both peripheral and central effects.

Systemic (40µg/kg; i.v) administration of secretin induces Fos in the central nucleus of the amygdala (CeA), area postrema (AP), bed nucleus of the stria terminalis (BNST), SON, dorsal motor nucleus of the vagus (DMNV) and medial region of the NTS (Goulet *et al.*, 2003). Systemic treatment with secretin (40 and 100µg/kg; i.p) induced Fos strongly in the CeA, AP, locus coeruleus (LC), NTS, external lateral subnucleus of the parabrachial nucleus (PBel) and weakly in the dorsal motor nucleus of the vagus (DMNV), arcuate nucleus (ARC) and Barrington's nucleus (Bar) (Yang *et al.*, 2004b). Central administration

of secretin (10 and 30µg; i.c.v) induced Fos expression in the dorsal vagal complex, NTS, AP, LC, PVN, medial and central amygdala (Welch *et al.*, 2003). Hence, secretin of central and/or peripheral origin may activate behavioural and visceral reflex regulatory circuits (Welch *et al.*, 2003).

5.4.3. Secretin actions in the SON

Central administration of secretin (1µg/rat; i.c.v) increased Fos expression in SON oxytocin and vasopressin neurones (peak at 5min after injection; returned to basal 60min after injection) and also dose dependently (0.1 -10µg) increased peripheral oxytocin and vasopressin release (Takayanaki and Onaka, 2007). Secretin (2-50µM) bath application increased the release of oxytocin and vasopressin from hypothalamic explants (Takayanaki and Onaka, 2007). Chu *et al* suggested that endogenously released secretin from the hypothalamus could possibly act via an autocrine pathway to modulate excitability of magnocellular neurones, playing a role in central stress responses (Chu et al., 2006a). In the same way, secretin is thought to stimulate *c-fos* and vasopressin expression and somato-dendritic release of vasopressin by magnocellular neurones (Chu et al., 2006a, b).

Systemically administered secretin might influence the activity of magnocellular neurones of the SON at least in four ways.

1. By crossing the blood-brain barrier and directly binding with secretin receptors in the SON.
2. By stimulating AP neurones directly via the deficient blood-brain barrier.
3. By stimulating vagal afferents and thus indirectly activating the brainstem neurones (NTS) that project to the SON.
4. By promoting HCO_3^- release into the intestinal lumen from the exocrine pancreas that accompanies transepithelial movement of Na^+ and K^+ to maintain electrical neutrality. This draws water from plasma into the intestine passively resulting in plasma hyperosmolality, and stimulates vasopressin release (Kitagawa *et al.*, 1990). Vasopressin in turn acts on ductal cells in the pancreas to reduce secretin-stimulated pancreatic secretion (Kitagawa *et al.*, 1990; Ko *et al.*, 1999).

Central administration of secretin (0.1 - 10µg; i.c.v) induced Fos expression in SON oxytocin and vasopressin neurones (Takayanagi and Onaka, 2007). However, behavioural effects induced by secretin have been found even with as little as 0.001µg secretin given by i.c.v. injection in mice (Babarczy *et al.*, 1995). With respect to peripheral administration, secretin-induced Fos expression has been studied in the rat hypothalamus after 40µg/kg (13µg/rat of 300g body weight) given intravenously (Goulet *et al.*, 2003). This dose is

equivalent to 0.02µg secretin entering the brain, as 0.12% of intravenous secretin enters the brain (Banks *et al.*, 2002). However, intravenous infusion of secretin at 0.1µg/kg h^{-1} is similar to the rate at which secretin is released after a lipid meal in the rat (Lu and Owyang, 1995). Hence, a dose of 0.1µg/rat (i.v) was chosen to study the effect of secretin on SON neurones to resemble closely the physiological level and route to the brain.

5.4.4. Importance of in vivo electrophysiology in studying actions of secretin

CCK, a peptide similar to secretin, with a circulating half-life of approximately 2min (Thompson *et al.*, 1975; Hoffmann *et al.*, 1993) increases the firing rate of SON oxytocin neurones immediately after administration with the rate reaching a peak within 5min and returning to basal level by 15min (Leng *et al.*, 1991). The half-life of endogenous circulating secretin is 4.1min (Kolts and McGuigan, 1977) and 1.5-3min in rats (de Smul et al., 1974; Lehnert et al., 1974; Tanaka and Katayama, 1984). However, Fos expression in the SON induced by secretin (40µg/kg; i.v) was similar at 60 and 120min and absent by 240min (Goulet *et al.*, 2003). Hence, the duration of neuronal electrical excitation is not clear with Fos studies. Moreover, Fos studies indicate the activity of cells but do not indicate the intensity of activation of individual cells. The 'real-time' activity of single cells can be best studied using electrophysiology. Fos studies show that the number of cells expressing Fos after secretin treatment is dose dependent (Yang *et al.*, 2004b). However, the intensity of activation of individual cells in response to different doses can be studied only through electrophysiology. Hence *in vivo* electrophysiological experiments were planned to study the duration and degree of any activation of SON oxytocin and vasopressin neurones upon systemic administration of secretin.

5.4.5. Mode of action of secretin on SON neurones

Systemically administered (i.p) secretin signals to the brain by interacting with secretin receptors on vagal afferents and on AP neurones outside the blood-brain barrier (Yang *et al.*, 2004b; Li *et al.*, 2005). Secretin binds to the NTS and other regions in brainstem, thalamus, hypothalamus and cortex with specificity and high affinity (Nozaki *et al.*, 2002). Secretin depolarizes NTS neurones *in vitro* through activation of a nonselective cationic conductance (Yang *et al.*, 2004a). Double immunostaining with Fos and TH in NTS neurones showed that a substantial population of TH positive neurones were Fos positive after secretin given by i.p. injection at 40µg/kg (Yang *et al.*, 2004b). NTS neurones relay signals received from the vagal afferents to higher brain structures via ascending adrenergic or noradrenergic pathways (Cunningham *et al.*, 1990). Secretin-induced increases in Fos positive cells in the NTS were abolished by subdiaphragmatic vagotomy (Yang *et al.*, 2004b); Fos positive cells were not only abolished in the NTS but also in pontine and limbic

nuclei (DMNV, LC, Bar, PBel and CeA) which suggested that the NTS conveys vagal afferent inputs to other nuclei through direct or indirect neural pathways (Yang *et al.*, 2004b). The SON receives excitatory noradrenergic inputs from the NTS (Alonso and Assenmacher, 1984). To investigate the involvement of adrenergic pathways in secretin-induced effects in SON neurones, α-1 adrenergic receptors were blocked using benoxathian hydrochloride (a competitive α-1 adrenergic antagonist) and the effect on secretin-induced responses in SON neurones were studied during the blockade.

5.4.6. Secretin-induced somato-dendritic oxytocin release from the SON

There have been few studies on actions of secretin on feeding behaviour, and these have not shown anorectic or satiety effects in rats. Administration of 0.1 -100 clinical units/kg i.p. secretin had no effect on sham feeding in rats (Lorenz et al., 1979), and 10nmol/kg i.p. secretin also had no effect on feeding in rats (Garlicki et al., 1990). However, in sheep, i.v. infusion of secretin (8 clinical units/kg per hour) reduced food intake by 38% and 0.5 units/kg per hour reduced intake by 12% (Grovum, 1981).

As oxytocin has central anorectic effects in rats (Olson et al., 1991a), it becomes of interest to test whether secretin increases central release of oxytocin. It has been proposed that oxytocin released from magnocellular neurone dendrites might act centrally to reduce appetite (Sabatier et al., 2007). Direct application of secretin (2-50μM) *in vitro* induces dendritic oxytocin release from the isolated SON (Takayanagi and Onaka, 2007). Here it was hypothesized that systemic secretin does not induce central oxytocin release from magnocellular dendrites because of lack of evidence for anorectic effects of secretin.

5.4.7. Role of secretin in osmoregulation

Secretin like immunoreactivity has been found abundantly in the posterior pituitary (Charlton et al., 1982). The expression of secretin and its receptor is significantly increased during dehydration (Chu et al., 2009). Chronic hyperosmolality as a result of water deprivation and 0.9% saline drinking also elevates plasma secretin level (Chu et al., 2009).

Recently, secretin mediated osmoregulation has shown to be one of the vasopressin independent mechanism regulating water balance (Cheng et al., 2009). Secretin receptors are found in the kidney (Ohta et al., 1992). In anaesthetized hydrated rats, secretin exhibits anti-diuretic action (Charlton et al., 1986). Secretin receptor null mice (SCTR-/-), which does not have any alteration in the transcription levels of vasopressin mRNA, develops polydipsia and polyuria (Chu et al., 2007). The presence of aquaporin tupte-2 (AQP_2) water channels in the apical membrane of the collecting tubules is essential for water reabsorption and urine concentration (Deen et al., 1994). Secretin induces the translocation of these channels to the apical membrane during water deprivation (Chu et al., 2009).

5.4.8. Secretin vs CCK: Similarities and differences

CCK and secretin are synergistic in their functions (Lee, 1979; Buchan *et al.*, 1993; Li, 2007). Pancreatic exocrine secretion after a meal is mainly stimulated by synergistic interaction between secretin and CCK (You *et al.*, 1983; Chey *et al.*, 1984). A high dose (100U/kg) of secretin was found to be synergistic with CCK for satiety (Lorenz *et al.*, 1979). However, Fos patterns in the brain induced by equimolar doses of CCK (15µg/kg; i.v) and secretin (40µg/kg; i.v) were different: Fos expression was more pronounced in the CeA and the AP after secretin than after CCK treatment (Goulet *et al.*, 2003). In contrast to secretin, CCK treatment led to a pronounced increase in Fos expression in the NTS, DMVN and PVN (Goulet *et al.*, 2003). The electrical response of SON vasopressin neurones to central CCK is excitatory and to intravenous CCK is either inhibitory or there is no effect (Renaud *et al.*, 1987). As central administration of secretin induces Fos in SON vasopressin neurones and increases peripheral release of vasopressin (Takayanagi and Onaka, 2007) and bath application of secretin induces vasopressin release in hypothalamic explants (Chu et al., 2006a), we aimed to see whether, like CCK, the peripheral administration of secretin inhibits or does not affect the activity of SON vasopressin neurones.

5.5. Methods

5.5.1. Experiment 1: Effect of systemic administration of secretin on the electrical activity of SON oxytocin and vasopressin neurones in urethane-anaesthetised fasted/unfasted virgin rats in vivo.

Urethane-anaesthetized fasted/unfasted rats were fitted with an i.v. cannula on the day of the experiment. The SON and neural stalk were exposed via the ventral transpharyngeal surgery. After a minimum of 10min period of recording the basal activity, the effect of i.v. administration of 0.01µg, 0.1µg or 1µg secretin in 100µl normal saline on the electrical activity of SON oxytocin neurones was recorded. In the rats fasted for 18h, the response to 0.1µg secretin of SON oxytocin neurones was recorded. The response of phasic and non-phasic vasopressin neurones to 0.1µg secretin was also recorded in unfasted and fasted virgin rats.

Groups:

1. Unfasted rats
   a. Oxytocin neurones:
      i. 0.01µg secretin: n = 4
      ii. 0.1µg secretin: n = 26
      iii. 1µg secretin: n = 3
   b. Vasopressin neurones:

i. 0.1µg secretin: Phasic: n = 11

ii. 0.1µg secretin: Non-phasic: n = 21

2. Fasted rats

a. Oxytocin neurones: 0.1µg secretin: n = 3

b. Vasopressin neurones: 0.1µg secretin: n = 5

i. Phasic firing neurones: n = 3

ii. Non-phasic firing neurones: n = 2

5.5.2. Experiment 2: Effect of systemic secretin administration on plasma oxytocin release in urethane-anaesthetised rats.

Urethane-anaesthetized virgin rats (n=6) were fitted with an i.v. cannula. After a minimum of 2h, a basal blood sample was taken. Subsequent sampling was done as per the following schedule.

Blood Sampling Schedule:

1. Sample 1 – Basal (2h after i.v. cannulation)
2. Sample 2 – 5min after vehicle (100µl saline i.v)
3. Sample 3 - 5min after secretin (0.1µg in 100µl saline; i.v)
4. Sample 4 – 10min after 0.1µg secretin
5. Sample 5 - 25 min after s0.1µg ecretin
6. Sample 6 - 5min after secretin (1µg in 100µl saline; i.v)
7. Sample 7 - 10min after 1µg secretin

5.5.3. Experiment 3. Effect of systemic administration of secretin on somato-dendritic oxytocin release from the SON in vivo in urethane-anaesthetized female rats.

The SON was exposed through the ventral transpharyngeal surgical procedure (see Section 2.3.1). The loop of a microdialysis probe was positioned on the ventral surface of the SON (see Section 2.4). The SON was dialysed with aCSF at a flow rate of 3µl/min using an infusion pump. Collection of basal microdialysate samples were begun an hour after initial dialysis. Secretin (1µg/rat) was administered i.v. and samples were collected on ice once every 30min for 2.5h. Subsequently, hypertonic aCSF (1M NaCl) followed by isotonic aCSF was microdialysed as a positive control for central oxytocin release, as an increase in SON oxytocin release is expected following hypertonic aCSF microdialysis and a rebound increase is expected following subsequent isotonic aCSF (Ludwig et al., 1994; Ludwig and Leng, 2006). The samples (90µl/tube) were stored at -20C until sent to Prof. Rainer Landgraf's Laboratory (Max Planck Institute of Psychiatry, Germany) for oxytocin RIA. Data from those rats that showed a positive response to hypertonic aCSF followed by

isotonic aCSF microdialyses were statistically analysed to seek evidence of secretin-induced central oxytocin release from the SON.

5.5.4. Experiment 4: Effect of i.c.v. infusion of benoxathian on secretin-induced excitatory electrical activity of SON oxytocin and vasopressin neurones in vivo.

The surgical procedure was carried out as per the procedure in i.v. and i.c.v. cannulated urethane-anaesthetized virgin rats. After an initial basal recording of firing rate of SON oxytocin (n=6) and vasopressin (n=3) neurones for a minimum period of 10min, 0.1µg secretin in 100µl normal saline was administered i.v. The same dose was repeated in some neurones to compare the responses so as to rule out receptor desensitization. Benoxathian was infused i.c.v. at a dose rate of 8µg/µl min^{-1}. Another secretin injection was repeated during the infusion. The firing rate was recorded throughout the experiment.

5.5.5. Experiment 5: Effect of benoxathian microdialysis onto the SON on secretin-induced excitatory electrical activity of SON oxytocin neurones in vivo.

The surgical procedure was carried out as per the procedure in i.v. cannulated urethane anaesthetized virgin rats. After an initial basal recording of firing rate of SON oxytocin neurones (n=3) for a minimum period of 10min, 0.1µg secretin in 100µl normal saline was administered i.v. When the effect of secretin subsided, microdialysis of benoxathian (2mM) was begun at a flow rate of 3µl/min. Another secretin injection was repeated during the microdialysis. The firing rate was recorded throughout the experiment.

5.6. Results

5.6.1. Effect of systemic administration of secretin on SON oxytocin neurones in unfasted virgin rats

Secretin (0.1µg/rat) was given intravenously after recording the basal firing-rate for a minimum period of 10min. Secretin excited all the SON oxytocin neurones tested (n=26; Fig. 5.1 and 5.2). The basal firing rate and the percentage increase in firing rate are negatively correlated (Fig. 5.2a). The number of spikes occurring within 0-0.5s after a spike is significantly increased after secretin administration which was reflected on the interspike interval histogram (Fig. 5.3). There was a significant positive shift in the hazard function (Fig. 5.4). The ratio of peak early (<0.07s) to mean late hazard (0.2-0.3s) 10min before (0.9 ± 0.05) and after secretin (0.9 ± 0.06) did not differ from each other (Fig. 5.5).

5.6.2. Effect of systemic administration of secretin on SON vasopressin neurones in unfasted virgin rats

The effect of systemic secretin administration (0.1µg/rat; i.v) was studied in 21 non-phasic and 11 phasic firing SON vasopressin neurones.

5.6.2.1. Non-phasic vasopressin neurones

Fourteen out of 21 non-phasic vasopressin neurones were excited (Fig. 5.6 and 5.7) while 6 out of 21 were inhibited (Fig. 5.8 and 5.9) and 1 out of 21 did not respond. There was no difference in basal firing rate among these neurones (P=0.3; t-test). On average, the basal firing rate of 6.3 ± 0.6 spikes/s was increased by 0.9 ± 0.2 spikes/s 4 min after secretin (n=21). However, the excitatory response observed 0-10min after secretin administration was not significantly higher compared to the basal period (Fig. 5.10).

5.6.2.2. Phasic vasopressin neurones

The response to systemic administration of secretin (0.1µg/rat; i.v) was tested in eleven phasically firing SON vasopressin neurones (Fig. 5.11). The average activity quotient increased and the mean interburst interval decreased 0-5min after secretin Fig. 5.12 and 5.13). The mean frequency within bursts remained unchanged (Fig. 5.14). Hence, systemic administration of secretin excited the phasic firing SON vasopressin neurones. This excitation did not reflect on the interspike interval histograms (Fig. 5.15a and b). There was no change in the shape of the hazard plot after secretin (Fig. 5.16).

5.6.3. Effect of systemic administration of secretin on SON oxytocin neurones: Dose-related responses

After recording basal firing rate for 10min, secretin was administered intravenously at three different dose rates: 0.01µg (n=4), 0.1µg (n=26) and 1µg (n=3) in 100µl normal saline; all doses were total per rat. Secretin-induced excitatory responses in SON oxytocin neurones (e.g. Fig. 5.17, 5.18 and 5.19) were dose dependent. The responses observed 0-10min after secretin between the three different groups were significantly different from each other (Fig. 5.20).

5.6.4. Plasma oxytocin release upon systemic administration of secretin in unfasted anaesthetized rats

Systemic secretin dose-dependently elevated the plasma oxytocin concentration in unfasted rats (Fig. 5.21). Secretin (0.1 and 1µg per rat) significantly elevated the oxytocin level compared to the basal and vehicle-induced change in the level. The increase in the oxytocin concentration following 1µg secretin was significantly higher than 0.1µg secretin.

5.6.5. Effect of systemic administration of secretin on somato-dendritic release of oxytocin from the SON in vivo in urethane-anaesthetized unfasted female rats

Four out of six rats that showed a positive response to hypertonic and subsequent aCSF microdialysis were statistically analysed to seek evidence of secretin-induced central oxytocin release from the SON (Fig. 5.22a). These rats also showed an evident excitatory response to systemic administration of secretin (1µg/rat) (Fig. 5.22b). The response was seen

only more than 1h after administration of secretin. On average, the excitatory response after secretin showed a trend to significance (Fig. 5.22c). Compared to peripheral oxytocin release following secretin, release at the SON is much delayed (Fig. 5.22d).

5.6.6. Effect of secretin administration on electrical activity of SON neurones in fasted virgin rats compared to unfasted virgin rats

5.6.6.1. SON oxytocin neurones

The average body weight, mean basal firing rate and secretin-induced change in the firing rate of SON oxytocin neurones in fasted virgin rats were not different from unfasted virgin rats (body weight: P=0.4, t-test; basal firing rate: P=0.8, t-test; secretin-induced change in the firing rate: P=0.9, t-test; Fig. 5.23).

5.6.6.2. SON vasopressin neurones

The average body weight of fasted and unfasted rats did not differ from each other (P=0.6; t-test). The response to systemic administration of secretin (0.1µg; i.v) was observed in 2 non-phasic and 3 phasic firing SON vasopressin neurones in fasted rats. One of the non-phasic vasopressin neurone was inhibited by secretin while the other did not respond (data not shown). In contrast, all the three phasic firing vasopressin neurones tested were excited by secretin; however, the excitation was not significant. There was no difference in basal and secretin-induced activity of phasic firing SON vasopressin neurones between fasted and unfasted rats (Fig. 5.24, 5.25 and 5.26).

5.6.7. Effect of i.c.v. benoxathian infusion and microdialysis of benoxathian onto the SON on secretin-induced excitation of SON oxytocin and vasopressin neurones

As the experiments with benoxathian microdialysis and i.c.v. infusion required successive treatments with same dose of secretin, in an initial experiment, the same dose of secretin was injected repeatedly and the responses were compared to examine the possibility of receptor desensitization. The responses to repeated injections of secretin were not different in both oxytocin (Fig. 5.27, 5.28 and 5.28a) and vasopressin neurones (Fig. 5.29 and 5.29a) tested.

5.6.8. Effect of i.c.v. infusion of benoxathian on secretin-induced excitation of SON oxytocin neurones

Six SON oxytocin neurones were tested for the involvement of NA pathway in secretin-induced excitation. The basal firing rate and percentage increase in the firing rate following secretin were negatively correlated (P=0.004; Pearson's correlation coefficient = -9.5). During benoxathian i.c.v. infusion (8µg/µl min^{-1}), the average basal firing rate of 5.8 ± 1.16 spikes/s was decreased by 29 – 100 % (28.83%, 38.67%, 58.49%, 79.26%, 100%, and 100%) in those 6 neurones studied. As per the negative correlation, lesser the basal firing

rate, greater should be the increase in firing rate following secretin. However, the secretin-induced excitatory response was also suppressed in all the 6 neurones by i.c.v. benoxathian infusion which reduced or abolished the basal firing rate (e.g. Fig. 5.30). The excitatory response 0-5min after secretin, before benoxathian i.c.v. infusion, was significantly greater than that during benoxathian (Fig. 5.31).

5.6.9. Effect of benoxathian i.c.v. infusion on secretin-induced excitation of phasic firing SON vasopressin neurones

Benoxathian i.c.v. infusion totally blocked the basal activity of 3 out of 4 phasic vasopressin neurones in 20min since it was begun (e.g. Fig. 5.32). The firing rate of one of the four neurones increased uncharacteristically following the infusion and hence it was excluded from the analysis. Benoxathian also blocked the excitatory response to secretin in all the three neurones tested (Fig. 5.33). After stopping the infusion, the cells started to resume their basal activity and upon secretin injection, elicited mild excitatory response.

5.6.10. Effect of benoxathian (2mM) microdialysis onto the SON on secretin-induced excitation of SON oxytocin neurones

The average basal firing rate before benoxathian microdialysis was 3.1 ± 0.5 spikes/s and it was reduced by 54.22%, 76.06% and 86.71% in the three neurones during benoxathian microdialysis. Benoxathian microdialysis also suppressed the secretin-induced excitatory response in all the three cells (Fig. 5.34).

5.7. Discussion

5.7.1. Responses of SON neurones to systemic secretin: Duration of action

Secretin clearly and consistently excited SON oxytocin neurones, and the activity returned to basal by 20min with doses up to 1µg i.v. The peak electrical activity observed 2min after intravenous secretin injection in this study correlates with the maximum blood concentration 2min post-dosing observed by Goulet *et al* (2003). The half-life of circulating endogenous secretin is only 1.5 - 3min in rats (de Smul et al., 1974; Lehnert et al., 1974; Tanaka and Katayama, 1984). Bath application of secretin to brain preparations *in vitro* resulted in increase in firing frequency of action potentials in whole cell current clamp recording of NTS that lasted for only 7-12min (Yang *et al.*, 2004a). However, secretin (40µg/kg; i.v) induced Fos expression in the SON was similar at 60 and 120min and absent by 240min (Goulet *et al.*, 2003). The expression of Fos for up to 120min after intravenous secretin could be due to the higher dose used, or more likely to the secretin-induced Fos expression in SON neurones lasting longer than the electrical activity.

The ratios of peak early to mean late hazard before and after secretin did not differ from each other suggesting that there is no change in the shape of the hazard after secretin

(Brown et al., 2008). A positive shift in the hazard function without a change in the shape of the hazard plot suggests that the increase in the probability of a spike firing might result from increased synaptic drive and/or a sustained depolarization upon which continued/increased synaptic drive is superimposed (Brown *et al.*, 2008).

5.7.2. Responses of SON neurones to physiologically similar dose

Secretin elicited robust excitatory responses in SON oxytocin and vasopressin neurones in pioneer studies in which higher doses were used (5, 10 µg i.v; data not shown). The dose was reduced gradually until it was found that doses less than 0.01µg were not effective. Secretin at a dose of 0.01µg i.v. was not always effective, while 0.1µg i.v. produced consistent excitation in SON oxytocin neurones. Thus a dose of 0.1µg i.v. secretin was used in further experiments. The dose chosen was nearly physiological as i.v. secretin at 0.1µg/kg h^{-1} is physiologically similar to the rate of postprandial release (Lu and Owyang, 1995).

Only 0.12% of intravenous secretin enters brain (Banks *et al.*, 2002), hence only 0.12ng of secretin out of the 0.1µg i.v. dose used in our study is expected to enter brain. This is the lowest systemic dose of secretin to be reported to have central effects. Behavioural effects were found even with as little as 0.001µg given by the i.c.v. route in mice (Babarczy *et al.*, 1995). Other studies have examined the secretin dose response relationship for induction of Fos expression in the CeA with doses starting from 0.4µg/kg i.v., but failed to find a significant increase in Fos at this dose, while Fos expression was significantly greater when the dose was increased 10-fold (Goulet *et al.*, 2003).

5.7.3. Dose-dependent responses of SON oxytocin neurones

Secretin dose-dependently excited the firing rate of SON oxytocin neurones. The excitation gradually subsided and returned to basal by 20min with all of the doses used. Systemic secretin dose-dependently increased the number of Fos expressing cells in the CeA (Goulet *et al.*, 2003 and Yang *et al.*, 2004b), suggesting that more cells are recruited to be activated as the dose increases. The results from the present study show that even the intensity of activation of single neurones is dose- dependent.

5.7.4. Secretin-induced increase in plasma oxytocin release

An increase in the electrical activity of SON oxytocin neurones represents increase in the peripheral release of oxytocin. This is further confirmed by the study on plasma oxytocin release following secretin administration. Systemic secretin dose-dependently increased the plasma oxytocin level in unfasted anaesthetized rats. Previously, Takayanagi and Onaka also reported dose-dependent increase in the plasma oxytocin release following central administration of secretin (0.1-10 µg; i.c.v) (Takayanagi and Onaka, 2007).

5.7.5. Systemic secretin-induced somato-dendritic oxytocin release from the SON

Secretin, given i.v, has been shown to reduce food intake in sheep (Grovum, 1981) but not in rats, when given i.p (Lorenz et al., 1979; Garlicki et al., 1990). From this perspective, it is interesting that systemic administration of secretin (1µg/rat) tended to increase release of oxytocin, an anorectic peptide, from the ventral surface of the SON. This finding indicates that further studies on the effect of intravenous secretin on food intake in rats are required.

Secretin (1µg/rat, i.v.) excited SON oxytocin neurones immediately after its administration and the response subsided within 30min. Similarly, secretin elevated plasma oxytocin concentration immediately after its administration. However, any SON release of oxytocin following secretin seems to be delayed for more than an hour. This pattern is similar to the peripheral and central oxytocin release profile observed after systemic osmotic stimulation (Ludwig et al., 1994).

The electrical activity is coupled with peripheral oxytocin release but is not always coupled with central somato-dendritic release from magnocellular neurones, which may be regulated independently (Ludwig et al., 1994; Ludwig and Leng, 2006). The increase in the intracellular Ca^{2+} level following action potential results in the activity-dependent exocytosis of small synaptic vesicles (SSV) at the axon terminals (Leng et al., 1999). This release is immediate as the SSV are aligned at the terminals ready to be released. On the contrary, the exocytosis of oxytocin-containing large dense core vesicles (LDCV) at the dendrites requires the mobilization of the vesicles from the reserve pool to the releasable pool, which is a slow process. However, if the intracellular Ca^{2+} concentration is increased by stimulating Ca^{2+} release from the endoplasmic reticulum, the LDCV in the reserve pool are mobilised to the releasable pool and these are docked at the membrane ready to be released upon subsequent electrical activity (Ludwig and Leng, 2006). This mechanism called ‘priming’ is also regulated by centrally released oxytocin which thus auto-regulates its own release (Ludwig and Pittman, 2003). The immediate activity-dependent peripheral oxytocin release and the subsequent dendritic oxytocin release at a later stage, following secretin administration, might be physiologically relevant: peripheral release might regulate the sodium balance on a moment-to-moment basis as the animal starts feeding; lack of central oxytocin release might promote feeding initially while the subsequent release after a period of time might gradually mediate satiety so that feeding stops.

Oxytocin release from the isolated SON after direct application of secretin *in vitro* (Takayanagi and Onaka, 2007) suggests direct action of secretin on secretin receptors on oxytocin neurones (Chu et al., 2006a). The delayed SON oxytocin release following

systemic secretin administration observed in this preliminary study possibly indicates an indirect effect via inputs to the SON, for example via vagal afferents and a brainstem pathway, by analogy with CCK (Onaka et al., 1995a) or possibly via arcuate nucleus POMC neurones, as α-MSH stimulates somato-dendritic oxytocin release from the SON (Sabatier et al., 2003a).

5.7.6. Secretin-induced responses in SON vasopressin neurones

Secretin is functionally related to CCK (Chey and Chang, 2001). Systemic administration of CCK does not increase the electrical activity or peripheral release of vasopressin neurones (Verbalis et al., 1986a; Verbalis et al., 1986b; Renaud et al., 1987). However, direct local application of CCK evokes vasopressin release from perfused hypothalamic-neurohypophysial explants (Jarvis et al., 1995). Central administration of secretin resulted in Fos expression in SON vasopressin neurones and increased peripheral release of vasopressin (Takayanagi and Onaka, 2007). Bath application of secretin onto hypothalamic explants induced vasopressin release (Takayanagi and Onaka, 2007). However, the response of SON vasopressin neurones to systemic administration of secretin was not previously known. The experiments here were aimed to see whether secretin-induced responses in SON vasopressin neurones are similar to those induced by CCK when administered intravenously, i.e. either inhibitory or without any effect on the firing rate in contrast to excitatory effect seen during central administration. Interestingly, systemic secretin either excited or inhibited non-phasic vasopressin neurones, with excitatory effect being predominant, and excited all of the phasic vasopressin neurones. Physiologically, the passive movement of water from plasma into the intestinal lumen following secretin-induced exocrine pancreatic secretion of HCO3- results in plasma hyperosmolality and stimulates vasopressin release (Kitagawa et al., 1990; Ko et al., 1999). However, it is surprising that secretin action on vasopressin neurones is immediate suggesting that in addition to plasma hyperosmolality, other direct/indirect means of secretin-induced activation is possible for SON vasopressin neurones.

5.7.7. Basal activity of SON oxytocin neurones in the fasted animals

In the unfed state, the activation of SON oxytocin neurones might be suppressed to avoid natriuresis and diuresis. This is supported by the observation that Fos expression has been found in the SON of fed but not unfed sheep (Chaillou *et al.*, 2000) and Fos expression in the SON in unfed rats was less than that in fed rats while feeding significantly increased Fos in SON in schedule-fed rats (Johnstone *et al.*, 2006). Hence, the basal activity of SON neurones was expected to be less in fasted rats. However, the basal activity of SON neurones was not different between fasted and unfasted rats in this study. This might be due to the less

number of observations made in the fasted group. On the other hand, food deprivation might act as a stress stimulus increasing the basal activity of oxytocin neurones to release the stress hormone, oxytocin. Moreover, secretin levels are increased in fasted state in humans and dogs (Mason *et al.*, 1979; Manabe *et al.*, 1987) which, if it is the same case in rats, might overcome reduction, if any, of the basal activity of SON neurones. In addition, it might also be due to the anaesthetic used in the study. Urethane, the anaesthetic that preserves physiological function in the oxytocin neurones, like the milk-ejection reflex, which is impaired by most anaesthetics, is the anaesthetic of choice for studying this system (Leng and Dyball, 1991). However, urethane activates the neurones via its mild hyperosmotic effect as the SON neurones are physiologically osmosensitive (Mason, 1980). Hence, the anaesthetic effects might block the difference, if any, between the basal activity of SON neurones in unfasted and fasted rats.

5.7.8. Secretin-induced activity of SON neurones in fasted animals

Secretin-induced activity of SON neurones is also not different between fasted and unfasted rats. This is in contrast to the lack of Fos expression seen in SON in fasted rats upon systemic administration of secretin (Goulet *et al.,* 2003). Secretin (40µg/kg; i.v) increased the number of Fos positive neurones in the SON in unfasted rats but there was no Fos expression in 18-20h fasted rats (Goulet *et al.,* 2003). This discrepancy in the secretin-induced activation of SON neurones in conscious and anaesthetized rats might be the effect of anaesthetic (Sabatier *et al.,* 2003a). The mild hyperosmotic effect of urethane activating the physiologically osmosensitive SON neurones might nullify the difference, if any, in the basal and secretin-induced activity of SON neurones in unfasted and fasted rats. However, it is possible that secretin excites oxytocin neurones with fasting having no influence at all.

5.7.9. Secretin-induced desensitization of secretin receptors

Desensitization of receptors to ligand stimulation is a phenomenon to protect cells from overstimulation which may result in cell damage (Siu *et al.*, 2006). The desensitization of secretin receptors remains controversial (Siu *et al.*, 2006). Chinese Hamster ovarian cells transfected with human secretin receptor were exposed to secretin and the extracellular acidification rate, an indicator of metabolic stimulation, was measured as a response to secretin (Ng *et al.*, 1999). When the cells were continuously exposed to secretin, the response (i.e. acidification) increased rapidly at first and then attained a plateau that suggested occurrence of desensitization limiting the maximal response (Ng *et al.*, 1999). However, when the cells were exposed to secretin repeatedly, the peak responses of the cells did not change significantly between exposures suggesting that the secretin receptor did not exhibit desensitization (Ng *et al.*, 1999). In the present study, excitatory responses to

repeated injections of secretin were tested in SON oxytocin and vasopressin neurones to rule out the possibility of receptor desensitization so that the same dose of secretin could be given repeatedly in further experiments. Repeated injections of secretin produced almost identical excitatory responses in both oxytocin and vasopressin neurones, hence receptor desensitization did not occur with the pattern of secretin exposure in the current experimental model, and this enabled us to conduct further experiments involving repeated injections of secretin.

5.7.10. Pathways involved in the systemic secretin-induced activation of SON neurones

The activation of SON neurones following systemic secretin administration could occur in two ways (Fig. 5.35):

1. Directly by the binding of secretin to secretin receptors on the SON neurones (Chu et al., 2006a) bypassing blood-brain barrier (Banks et al., 2002).
2. Indirectly by the activation of vagal afferents and subsequently brainstem nuclei.

Secretin activates vagal afferents (Li et al., 2005) and depolarizes neurones in the NTS (Yang et al., 2004b). Subdiaphragmatic vagotomy abolishes Fos expression induced by systemic secretin in the NTS but it was maintained in the AP (Yang et al., 2004b) suggesting that secretin may act directly on the AP neurones and indirectly, via vagal afferents, on the NTS neurones. Excitatory noradrenergic neurones of NTS (A2 group) project to the SON (Alonso and Assenmacher, 1984). The AP also has efferent projections to the SON via the A1 cell group (van der Kooy and Koda, 1983; Shapiro and Miselis, 1985; Johnson and Gross, 1993) and lateral parabrachial nucleus (Fulwiler and Saper, 1984; Shapiro and Miselis, 1985). The AP and NTS maintain reciprocal connections (Shapiro and Miselis, 1985; Ferguson, 1991; Hay and Bishop, 1991a, b), hence afferent input to the AP may be relayed first to the NTS and then conveyed to the SON or vice versa (Carlson *et al.*, 1998).

Systemic administration of CCK activates gastric vagal afferents and hence activates oxytocin neurones probably via the NTS (Verbalis et al., 1986a; Renaud et al., 1987; Onaka et al., 1995a). To test whether secretin induced excitation of SON oxytocin neurones also involves a noradrenergic pathway, we studied the effect of secretin during benoxathian (α-1 adrenergic antagonist) i.c.v. infusion and during microdialysis onto the SON.

Intracerebroventricular benoxathian infusion has been shown to successfully block the excitatory response of SON oxytocin neurones to CCK with an infusion rate of 5.3µg/min (Brown *et al.*, 1998). A benoxathian infusion rate of 4µg/min was found to be ineffective in reducing the basal firing-rate of SON neurones in this study (Fig. 5.30). Hence, benoxathian was infused i.c.v. at a rate of 8µg/min. Benoxathian significantly reduced the basal firing rate and secretin-induced excitation of SON oxytocin neurones. Hence

adrenergic pathways are involved in the excitation of SON oxytocin neurones induced by systemic secretin. This is in accordance with the positive shift in the hazard without change in the shape of the hazard plot of SON oxytocin neurones after secretin administration affirming that secretin merely increases the excitatory inputs without altering the intrinsic properties of the membrane.

Intracerebroventricular administration of benoxathian totally inhibited the basal firing and completely abolished the secretin-induced excitatory response in phasic vasopressin neurones. Hence it appears that noradrenaline facilitates the basal firing as well as secretin-induced excitation through α-1 adrenergic receptors in the phasic vasopressin neurones.

Though the results of this i.c.v. infusion study suggest the possibility of involvement of noradrenergic pathways in the secretin induced excitation of SON oxytocin neurones, it does not specify the role of excitatory noradrenergic projections from A2 group of NTS. Hence, benoxathian was microdialysed on the surface of the SON so as to block the local noradrenergic inputs which is predominantly from the NTS.

Microdialysis application of benoxathian to the SON also reduced the basal firing rate of the SON oxytocin neurones tested by 72.3 ± 11.7%. Previously, oxytocin neurone firing rate has been to be reduced by 53% after benoxathian microdialysis (Brown *et al.*, 1998). Hence, α1 adrenoceptors, and NA, at least in part, facilitate tonic excitation of SON oxytocin neurones. Technically, the reduction in the basal firing rate confirmed that benoxathian diffuses out of the microdialysis probe on to SON neurones, presumably blocking tonic activation via α1 adrenoceptors, and validating the whole experimental preparation suitable to carry out further experiments testing the effect of secretin during benoxathian microdialysis. Benoxathian microdialysis suppressed the secretin-induced excitatory response in all three SON oxytocin neurones tested suggesting that secretin-induced excitation of SON oxytocin neurones is via α1 adrenoceptors, mediated presumably by NA inputs from the NTS.

5.7.11. Secretin-induced oxytocin release: physiological implications

5.7.11.1. Feedback and feed-forward effects?

The results from this study suggest that some of the effects of secretin might, at least in part, be mediated through the activation of SON neurones and thus the release of oxytocin and possibly vasopressin. Secretinergic cells are found in the mPVN and SON (Welch *et al.*, 2004). SON neurones activated by secretin might even represent secretinergic neurones activated to modulate the somato-dendritic release of oxytocin, vasopressin or even secretin. Interestingly, oxytocin receptors are expressed in the intestine (Welch et al., 2009). Hence,

secretin-induced release of peripheral oxytocin might feed-forward to modulate the secretion of secretin from the duodenal S-cells.

5.7.11.2. Gastroprotection?

Secretin and oxytocin have many regulatory roles in common (Fig. 5.36). Physiologically, intra-duodenal acidification is the only well established stimulus for secretin release (Oektedalen *et al.*, 1982). In addition, stress-related gastrin and gastric acid secretion also act as an acute stimulus for the release of secretin (Li *et al.*, 1998). Secretin is a gastro-protective hormone (Bayliss and Starling, 1902). It prevents stress-induced gastric bleeding in the gastric mucosa in the rat (Murakami *et al.*, 1985). Impaired secretin release and reduced duodenal S-cells have been documented in peptic ulcer patients (Love, 2008a, b). Similarly, oxytocin showed significant antisecretory and antiulcer activity in rats (Asad *et al.*, 2001). It is possible that gastro-protective properties of secretin are complemented by secretin-induced oxytocin secretion.

5.7.11.3. Gastric motility?

Secretin receptors are localized on the longitudinal and circular muscle layers of the rat forestomach (Steiner *et al.*, 1993). The inhibitory effect of secretin on gastric emptying might be produced through direct action of secretin on secretin receptors (Zhou and Wang, 1990; Steiner et al., 1993). However, administration of secretin delays gastric emptying and inhibits gastric motility via a vagal afferent pathway originating from the gastroduodenal mucosa (Raybould and Holzer, 1993; Lu and Owyang, 1995). This pathway might culminate in the activation of oxytocin neurones and the release of oxytocin. Oxytocin inhibits gastric emptying and gastrointestinal transit in rats via a mechanism involving CCK stimulation and CCK1 receptor activation (Wu *et al.*, 2002; Wu *et al.*, 2003; Ohlsson *et al.*, 2006; Wu *et al.*, 2008). Oxytocin excited almost all of the gastric-related NTS neurones (McCann and Rogers, 1990). Hence, a synergistic action is possible for secretin and oxytocin to govern gastric motility.

5.7.11.4. Postprandial exocrine pancreatic secretion?

Postprandial release of oxytocin stimulates the release of atrial natriuretic peptide (ANP) (Haanwinckel *et al.*, 1995). ANP release also increases basal and hormone (CCK and secretin) evoked exocrine pancreatic secretion in a dose dependent manner (Sabbatini *et al.*, 2003). Hence it is possible that postprandial secretin induces release of oxytocin which in turn triggers ANP release to augment exocrine pancreatic secretion. In addition, oxytocin receptors are found in a rat pancreatic cell line (Jeng *et al.*, 1996). Oxytocin increases the release of insulin and glucagon from the endocrine pancreas (Bjorkstrand *et al.*, 1996), both of which increase the secretion from the exocrine pancreas (Ferrer *et al.*, 2000). Hence

secretin-induced oxytocin release might also augment exocrine pancreatic secretion through insulin and glucagon.

5.7.11.5. Postprandial natriuresis?

Postprandially released oxytocin promotes natriuresis and diuresis (Haanwinckel *et al.*, 1995). A secretin-oxytocin-ANP (or secretin-ANP) pathway might, if such a pathway exists, also govern post-prandial natriuresis. As ANP and oxytocin are colocalized in some hypothalamic magnocellular neurones (Jirikowski *et al.*, 1986), secretin-induced excitation of oxytocin neurones might release ANP, in addition to the release of oxytocin. The oxytocin-mediated natriuretic mechanisms are essential during the post-prandial period to regulate sodium balance.

5.7.11.6. Antidiuresis?

Recently, secretion has shown to be involved in osmoregulation. Dehydration increases the expression of secretin and its receptor in hypothalamic neurones (Chu et al., 2009) and water deprivation and saline drinking elevate plasma secretin level (Chu et al., 2009). Secretin receptors are found in the kidney (Ohta et al., 1992) and in anaesthetized hydrated rats, secretin exhibits anti-diuretic action (Charlton et al., 1986). Secretin receptor null mice (SCTR-/-), which does not have any alteration in the transcription levels of vasopressin mRNA, develops polydipsia and polyuria (Chu et al., 2007). Hence, secretin mediated osmoregulation is likely to be one of the vasopressin independent mechanism regulating water balance (Cheng et al., 2009).

Depending on the hydration status of the animal, oxytocin could be either diuretic or anti-diuretic. In Brattleboro rats, which congenitally lack vasopressin, the symptoms of diabetes mellitus were reversed by oxytocin infusion (Lyness et al., 1985). This anti-diuretic effect of oxytocin is mediated by V_2 receptors in the kidney (Li et al., 2008). Hence, anti-diuretic effects of secretin and oxytocin might complement each other, especially during periods of reduced water intake (e.g. fasting), during ingestion of dry feed or during excessive salt intake.

5.7.11.7. Energy homeostasis during starvation?

Secretin level in plasma dramatically increases from day 1 to 3 of fasting in humans and dogs (Mason *et al.*, 1979; Manabe *et al.*, 1987). This increment is several times higher than that observed after intraduodenal acid load and can be suppressed by i.v. glucose or refeeding (Thuesen *et al.*, 1987). *In vitro* experiments have shown that secretin stimulates lipolysis (Lazarus et al., 1968; Rudman and Del Rio, 1969; Rodbell et al., 1970), glycogenolysis (Matsumura *et al.*, 1977) and gluconeogenesis (Matsumura *et al.*, 1977). Similarly, oxytocin levels are significantly increased after short term food deprivation, and

insulin-induced hypoglycaemia elevates plasma oxytocin levels in female and male rats (Bjorkstrand *et al.*, 1992). In hepatocytes from starved rats, oxytocin stimulates gluconeogenesis from lactate by 25-50% (Hems *et al.*, 1978; Whitton *et al.*, 1978). Hence, during periods of hypoglycaemia, secretin could also act through oxytocin to elevate glucose levels. Interestingly, however, secretin did not stimulate Fos expression in the SON in fasted rats (Goulet *et al.*, 2003).

5.7.11.8. Secretin-induced central oxytocin release and regulation of appetite?

The evidence for the involvement of secretin in appetite regulation, such as food intake and satiety, is lacking in the literature. This study showed that secretin induces somato-dendritic oxytocin release from the SON. Central oxytocin is anorectic (Olson et al., 1991a) but it is not known whether secretin induces central oxytocin release during the postprandial period to mediate satiety. Systemic administration of secretin (0.1-100 clinical units/kg; i.p) failed to elicit satiety in the sham feeding rat (Lorenz *et al.*, 1979) and 10nmol/kg i.p. secretin also had no effect on feeding in rats (Garlicki et al., 1990), unlike CCK which is a satiety peptide (Antin *et al.*, 1975).

5.7.11.9. Secretin-induced central oxytocin release and regulation of social behaviour?

Cyclosporine, an immunosuppressive drug, disrupts social behaviour by reducing the release of serotonin and dopamine in the prefrontal cortex (Sato et al., 2007). Centrally administered secretin (1µg/rat; i.c.v) partially restored abnormal social interaction after cyclosporine in rats (Takayanagi and Onaka, 2007). There is evidence that secretin is effective in some people with autistic spectrum disorders (Horvath et al., 1998; Lamson and Plaza, 2001; Alamy et al., 2004). There are some limited or mixed results available in the literature as well (Chez *et al.*, 2000; Coniglio *et al.*, 2001; Kern *et al.*, 2002; Pallanti *et al.*, 2005; Ratliff-Schaub *et al.*, 2005). Controversially, in several other studies, secretin produced no effect or brought about deterioration of behaviour and communication capabilities in autistic patients (Sandler *et al.*, 1999; Dunn-Geier *et al.*, 2000; Corbett *et al.*, 2001; Lightdale *et al.*, 2001; Owley *et al.*, 2001; Roberts *et al.*, 2001; Robinson, 2001; Carey *et al.*, 2002; Molloy *et al.*, 2002; Sponheim *et al.*, 2002; Unis *et al.*, 2002; Levy *et al.*, 2003).

Two single nucleotide polymorphisms in oxytocin receptors have been associated with autism in the Chinese Han population (Wu *et al.*, 2005). Post mortem analyses of a developmental hyperserotonemia (DHS) model of autism in rats revealed that there is a loss of oxytocin neurones in the PVN (McNamara *et al.*, 2008). Repetitive behaviour in autistic spectrum disorders was partially ameliorated by synthetic oxytocin infusion (Hollander *et al.*, 2003). Both secretin and oxytocin have been implicated in the treatment of autistic

spectrum disorders (Welch and Ruggiero, 2005; Welch et al., 2005). This reinforces the idea that the effects of secretin might be mediated, at least in part, through oxytocin.

5.7.12. Secretin-induced vasopressin release: physiological implications

Plasma hyperosmolality following secretin release, which occurs due to the passive movement of water from the plasma into the intestine following bicarbonate secretion, stimulates vasopressin release (Kitagawa et al., 1990; Konturek et al., 2003a, b) which in turn acts on ductal cells in the pancreas to reduce secretin-stimulated pancreatic secretion (Kitagawa *et al.*, 1990; Ko *et al.*, 1999). In addition to this slow effect, secretin treatment results in rapid release of vasopressin into the plasma (Takayanagi and Onaka, 2007; Chu et al., 2009). Vasopressin is antidiuretic (Abboud et al., 1990). In addition to being one of the vasopressin-independent mechanisms by itself (Cheng et al., 2009), secretin is likely to regulate water balance via vasopressin.

5.8. Conclusion

Systemic administration of secretin elicited a dose-dependent excitatory firing rate response in SON oxytocin neurones and a dose-dependent increase in plasma oxytocin release. Systemic secretin seems to increase somato-dendritic release of oxytocin from the SON. The electrophysiological response of vasopressin neurones to systemic secretin was predominantly excitatory. Noradrenergic pathways, possibly involving the NA-ergic projections from the brainstem neurones to the SON, mediate the secretin-induced excitatory effects via α-1 adrenoceptors in SON oxytocin and vasopressin neurones. Fasting did not alter the response of SON neurones to secretin. The influence of the gut peptide secretin on the electrical activity of SON neurones suggests that the SON is important in the regulation of brain-gut axis. Secretin and oxytocin have several physiological functions in common or linked, hence it is possible that the central and peripheral physiological effects of secretin are mediated, at least in part, through oxytocin.

CHAPTER VI

CHOLECYSTOKININ (CCK)

6.1. Introduction

Cholecystokinin (CCK) is synthesized in the I-cells of the intestinal mucosa (Polak et al., 1975) and is secreted when food enters the small intestine from the stomach. It stimulates enzyme secretion from the exocrine pancreas to digest fat, protein and carbohydrates. Peripheral CCK also mediates satiety (Liddle, 1997).

Systemic administration of CCK increases the electrical activity of SON oxytocin neurones in urethane-anaesthetized rats and increases the peripheral release of oxytocin in conscious and anaesthetized rats (Renaud et al., 1987; Hamamura et al., 1991). Although HPA axis and oxytocin responses to various stimuli are attenuated in late pregnancy (Russell et al., 2008), the CCK-induced excitatory response of SON oxytocin neurones in late pregnant rats is not different from virgin rats, except importantly, that the response is enhanced by naloxone only in late pregnant rats (Douglas et al., 1995). As fasting is a stressor and oxytocin is a stress hormone in the rat (Lang et al., 1983), increased basal and CCK-induced activity of oxytocin neurones is expected upon fasting. In addition, as leptin antagonises HPA responses to many stressors, including insulin-induced hypoglycaemia and fasting (Gaillard et al., 2000; Giovambattista et al., 2000), oxytocin neuronal responses to CCK can be expected to be augmented in fasted animals because fasting reduces endogenous levels of leptin (Ladyman and Grattan, 2004). On the other hand, homeostatic mechanisms might suppress CCK-induced excitation of oxytocin neurones to conserve the sodium level during fasting when the salt intake is less. Hence, it will be interesting to study the excitatory responses of SON oxytocin neurones following CCK administration in fasted virgin and pregnant rats and to compare these with the responses in unfasted rats.

Synergy between leptin and CCK has been observed in food intake and body weight regulation (Barrachina et al., 1997; Matson et al., 1997; Matson and Ritter, 1999; Matson et al., 2002). It is not known whether systemic leptin potentiates CCK-induced oxytocin neuronal excitation. The models of fasting and pregnancy, in which the endogenous leptin levels are decreased and increased, respectively, also provide a means to study this interaction between leptin and CCK.

As CCK is synergistic with secretin (You et al., 1983; Chey et al., 1984) and closely interacts with leptin (Barrachina et al., 1997) and all these peptides are released during the postprandial period (Schafmayer et al., 1978; Saladin et al., 1995), it was considered of interest to compare the responses of SON oxytocin and vasopressin neurones to CCK with

the responses induced by secretin and leptin to analyse the effectiveness of the peptides and to understand their influence on postprandial oxytocin/vasopressin secretion.

6.2. Hypotheses

1. CCK-induced excitation of SON oxytocin neurones are altered during fasting in virgin and pregnant rats.
2. Systemic leptin potentiates the CCK-induced excitatory responses of SON oxytocin neurones.

6.2.1. Objectives

1. To study the electrophysiological responses of SON oxytocin neurones *in vivo* after systemic administration of CCK in fasted urethane-anaesthetized virgin and pregnant rats and to compare with unfasted virgin and pregnant rats.
2. To study whether systemic administration of leptin potentiates the CCK-induced excitatory electrophysiological responses of SON oxytocin neurones in unfasted virgin rats.
3. To compare the electrophysiological responses of SON oxytocin and vasopressin neurones in unfasted/fasted virgin rats to systemic administration of secretin and leptin with the responses to CCK administration.

6.3. Background

6.3.1. Cholecystokinin

Cholecystokinin (CCK) was discovered in 1928 by Ivy and Oldberg during studies on hormonal mechanisms involving gallbladder contraction in dogs (Ivy and Oldberg, 1928). As similar intestinal extracts also stimulated pancreatic enzyme secretion, the name 'pancreozymin' was proposed (Harper and Raper, 1943). Purification and amino acid sequencing of the active substance showed that CCK and pancreozymin are the same and hence it goes now by the name cholecystokinin (Mutt and Jorpes, 1968).

The endocrine I-cells of the intestinal mucosal epithelium, that produce CCK, are concentrated in the duodenum and proximal jejunum (Polak et al., 1975). The release of CCK is stimulated by ingestion of food, with fats and protein the most potent secretagogues, and carbohydrates the least potent (Liddle, 1997). CCK secretion is initiated when the food enters the small intestine from the stomach and continues until protein, fat and their metabolites have passed the upper small intestine (Liddle, 1997). Apart from stimulating exocrine pancreatic enzyme secretion and gall bladder contraction, CCK also stimulates intestinal peristalsis and inhibits gastric emptying (Liddle, 1997).

6.3.2. Molecular forms of CCK

CCK is a heterogeneous hormone and is present in different molecular forms in mammals: CCK-83, CCK-58, CCK-39, CCK-33, CCK-22, and CCK-8 (Wang and Cui, 2007). The post-translational modification of the *CCK* gene product, preprocholecystokinin, results in CCK composed of varying numbers of amino acids. Originally, CCK was purified as a 33-amino acid peptide from the porcine intestine (Mutt and Jorpes, 1968). Since the discovery of CCK, multiple molecular forms have been identified in the intestine, brain, and the circulation of several species. The CCK octapeptide (CCK-8), consisting of the carboxy-terminal 8 amino acids of CCK, is the most potent biologically active small peptide form of CCK (Nakajima, 1973), and is also the dominant form in the rat brain (Lamers et al., 1980). However, CCK-58 seems to be the only major circulating form of CCK in the rat and other smaller forms arise due to degradation of CCK-58 during sample processing (Reeve et al., 2003).

The CCK in plasma contains a mixture of CCK molecules such as CCK-58, CCK-33, CCK-22, and CCK-8 (Beinfeld, 2003; Rehfeld, 2004). Different studies have reported different concentrations of plasma CCK before and after feeding in the rat [1.9 ± 0.3 *vs.* 13.4 ± 3.8 pM (Douglas et al., 1990), 2.5 *vs.* 9.7 ± 1.6 pM (Douglas et al., 1988), 0.5 ± 0.2 *vs.* 7.9 ± 1.9 pM (Liddle et al., 1986), 0.85 ± 0.1 *vs.* 8.2 ± 1.1 pM (Linden et al., 1989a), 2.5 ± 0.3 *vs.* 8.9 ± 0.6 pM (Sharara et al., 1993)]. Overall, before feeding, plasma CCK concentration

was found to be in the range of 0.5 ± 0.2 to 2.5 ± 0.3 pM and after feeding, it increased to 4.4 ± 0.8 to 13.4 ± 3.8 pM (Wang and Cui, 2007).

6.3.3. CCK in the CNS

CCK is also synthesized, stored and released in the CNS (Crawley, 1985). It is one of the most abundant peptides in the cerebral cortex, striatum and hippocampus (Beinfeld, 2001) and is present in moderate quantities in hypothalamic nuclei (Beinfeld et al., 1981b). The posterior pituitary receives CCK-containing fibres from the hypothalamic magnocellular nuclei (Beinfeld et al., 1981a). CCK co-exists with dopamine in some of the mesencephalic neurones and with oxytocin in some of the hypothalamic neurones (Vanderhaeghen et al., 1981). CCK modulates dopaminergic neurotransmission and has been implicated in dopaminergic regulation (Crawley, 1991). The blood-brain barrier completely blocks passage of CCK from the gut to the brain, although the movement of CCK from the CSF to the blood is controversial (Crawley, 1985; Zhu et al., 1986).

6.3.4. CCK receptors

CCK acts via its G-protein coupled receptors (GPCRs). Two CCK receptor subtypes have been identified - CCK_A (CCK_1) and CCK_B (CCK_2) receptors (R) - on the basis of differences in the affinity for the structurally and functionally related family of peptides, differences in the affinity for ligands with sulphation at the tyrosyl residues, differences in the responses to specific antagonists and differences in the tissue distribution (Miyasaka and Funakoshi, 2003). The CCK_AR binds sulphated CCK with higher affinity than gastrin, whereas the CCK_BR binds sulphated and non-sulphated CCK and gastrin with almost the same affinity (Jensen et al., 1989). The CCK_AR is predominantly found in the GI system and a few areas in the CNS whereas CCK_BR is predominantly found in the CNS and some areas in the GI system (Wank, 1995).

6.3.5. Role of vagal afferents in CCK-mediated effects

CCK-induced exocrine pancreatic secretion occurs via two mechanisms:

1. Activation of CCK_AR on the exocrine pancreas (Zhou et al., 1995).
2. Neural mediation involving activation of CCK_AR on vagal afferents.

CCK receptors are expressed in vagal afferents from gut (Li et al., 1997; Patterson et al., 2002). Bilateral vagotomy, pretreatment with atropine or hexamethonium or perivagal treatment with capsaicin completely abolished exocrine pancreatic secretion in response to low doses of CCK, suggesting that CCK at physiological levels stimulates pancreatic enzyme secretion via a capsaicin-sensitive afferent vagal pathway originating from the gastroduodenal mucosa (Li and Owyang, 1993; Schwartz et al., 1993). CCK-induced

stimulation of vagal afferents opposes the inhibitory effects of ghrelin on exocrine pancreatic secretion which is also mediated via the vagal pathway (Kapica et al., 2006).

6.3.6. CCK and satiety

Afferent axons of the vagus also mediate the satiety effects of peripheral CCK (Smith et al., 1985; Walls et al., 1995) via the low-affinity CCK_AR in the vagus (Li et al., 1997). Systemic administration of CCK results in Fos expression in the NTS (Rinaman et al., 1995), primarily in regions where vagal afferent fibres terminate (Fraser and Davison, 1992). CCK acts directly on the AP neurones because of the deficient blood-brain barrier (Fraser and Davison, 1992). 70% of the CCK-activated neurones in the NTS project to the PVN neurones (Rinaman et al., 1995) which probably are oxytocinergic (Blevins et al., 2003). However, the downstream pathways involving CCK-induced satiety are not very clear because, in addition to the activation of the NTS and AP, peripheral CCK administration results in Fos expression in many areas in the brain such as the LC, subcoeruleus nucleus (SC), dorsal vagal complex, external subdivision of the lateral parabrachial nuclei (PBel), BNST, CeA, DMH, PVN and SON (Fraser and Davison, 1992; Li and Rowland, 1995; Monnikes et al., 1997; Eckel et al., 2002; Kobelt et al., 2006). CCK-induced *c-fos* mRNA has also been observed in the brainstem (NTS and AP), amygdala (CeA) and hypothalamus (PVN and SON) (Hamamura et al., 1991; Day et al., 1994). However, the VMH and ARC did not show any Fos expression following peripheral administration of CCK (Kobelt et al., 2006). The induction of Fos in the brain and brainstem involves activation of CCK_AR (Monnikes et al., 1997) and capsaicin sensitive vagal afferents (Monnikes et al., 1997; Sayegh and Ritter, 2000). Destruction of NA neurones in the NTS attenuates CCK-induced satiety (Rinaman, 2003). Recently, bilateral ablation of the SON has been found to abolish CCK-induced satiety (Takashi Higuchi, *personal communication*).

Centrally released CCK might also contribute to satiety because CCK is released from hypothalamic neurones during feeding in primates and rats (Schick et al., 1987; De Fanti et al., 1998). Moreover, centrally administered CCK results in satiety (Shiraishi, 1990).

6.3.7. Pathways involved in CCK-induced SON neuronal responses

Systemic administration of CCK increases the electrical activity of SON oxytocin neurones in urethane-anaesthetized rats and increases the peripheral release of oxytocin in conscious and anaesthetized rats (Renaud et al., 1987; Hamamura et al., 1991). CCK-induced *c-fos* mRNA expression in the SON and peripheral release of oxytocin in conscious rats is abolished by gastric vagotomy (Verbalis et al., 1986a).

Although the SON neurones possess CCK receptors (Day et al., 1989), peripheral CCK does not cross the blood-brain barrier (Crawley, 1985; Zhu et al., 1986). Hence,

peripheral CCK activates the NTS through the activation of vagal afferents via the CCK_AR, (Glatzle et al., 2001), subsequently resulting in the activation of SON neurones (Onaka et al., 1995a). However, AP neurones are directly activated by CCK (Fraser and Davison, 1992). The A2 cell group within the NTS and the Al cell group within the VLM are the sources of direct NA projections to the SON and the PVN (Sawchenko and Swanson, 1982). The axon terminals of the NA neurones in the A2 cell group make synaptic contact with the SON neuronal cell bodies (Alonso and Assenmacher, 1984). Peripheral CCK injections are followed by a marked increase in the NA release within the dorsal oxytocin-rich regions of the SON as measured by microdialysis (Kendrick et al., 1991; Tobin et al., 2009). In addition, destruction of NA fibres in the vicinity of the SON significantly decreased the CCK-induced Fos expression in the SON (Onaka et al., 1995a). The NA terminals mediating CCK-induced excitation of SON oxytocin neurones are sensitive to opioids because direct morphine administration into the SON blocks the NA release in the SON and systemic morphine administration blocks CCK-induced Fos expression in the SON and oxytocin release (Onaka et al., 1995b).

Although a projection from the A2 cell group of the NTS to the oxytocin and vasopressin neurones of the SON has been established, this projection does not contribute directly to the control of SON vasopressin neuronal activity (Day, 1989). Rather, NTS stimulation excites SON vasopressin neurones due to activation of a relay projection through the A1 cell group of the caudal VLM (Day, 1989; Day and Sibbald, 1989). This pathway is thought to be involved in the stimulation of vasopressin secretion by haemorrhage and its inhibition by baroreceptor activation (Day, 1989). Systemic CCK administration does not increase Fos expression in the NA neurones in the A1 region of the brainstem that project to the SON (Onaka et al., 1995a), inhibits NA release into the vasopressin-rich region of the SON (Tobin et al., 2009) and does not increase either vasopressin release or vasopressin neuronal activity (Verbalis et al., 1986a; Renaud et al., 1987).

However, direct application of CCK is excitatory to both oxytocin and vasopressin neurones of the SON (Brown et al., 2000a). *In vitro* application of CCK depolarizes SON neurones (Jarvis et al., 1992) and increases the release of oxytocin and vasopressin from isolated neurohypophysial nerve terminals and from hypothalamo-neurohypophysial explants (Bondy et al., 1989; Jarvis et al., 1995).

6.3.8. CCK and leptin interaction

Peripheral administration of CCK reduces meal size in several species (Woods et al., 1981). Interestingly, recent studies on meal patterns after leptin administration show that leptin-induced reduction in food intake is targeted specifically on reducing the amount rather

than changing the frequency of meal (Eckel et al., 1998; Flynn et al., 1998; Kahler et al., 1998). Hence, through within-meal satiety peptides like CCK, long-term satiety signals like leptin regulate food intake (Emond et al., 1999; Wang et al., 2000).

Synergy between leptin and CCK has been observed both in short term responses such as food intake and long term responses such as body weight regulation (Barrachina et al., 1997; Matson et al., 1997; Matson and Ritter, 1999; Matson et al., 2002). Leptin limits food intake on a meal-to-meal basis by regulating NTS and AP responses to CCK (Morton et al., 2005). Peripheral injections of a combination of CCK and leptin, at doses inefficient when given singly, suppressed food intake by 80% in fasted lean mice (Barrachina et al., 1997). Leptin injected with CCK increased Fos expression in the PVN by 71% in 24h fasted lean mice (Wang et al., 1998). The mechanism by which CCK reduces body weight is partially dependent on central leptin pathways (Merino et al., 2008). In turn, CCK promotes leptin transport across the blood-brain barrier (Cano et al., 2008) and reduced CCK signaling influences the action of leptin (Barrachina et al., 1997).

In summary, administration of leptin augments responses to CCK (Barrachina et al., 1997; Wang et al., 1998; Emond et al., 1999) while genetic or fasting-induced reduction in leptin signaling diminishes CCK-induced responses (McLaughlin and Baile, 1980; Niederau et al., 1997; McMinn et al., 2000). However, it is not known whether CCK-induced excitatory responses of SON oxytocin neurones are potentiated by systemic leptin administration. During pregnancy, the circulating level of leptin is increased (Kawai et al., 1997) and during fasting, it is decreased (Ladyman and Grattan, 2004). Hence, pregnancy and fasting are excellent models to study the interaction of CCK and leptin on oxytocin neurones.

6.3.9. CCK-induced SON oxytocin neuronal responses during pregnancy

During pregnancy, alterations in the CCK-induced excitatory responses of SON oxytocin neurones are expected for at least four reasons:

1. The plasma volume is elevated during pregnancy (Brown and Pike, 1960; Rosso and Streeter, 1979). Oxytocin release and hence natriuresis are expected to be attenuated during pregnancy to maintain the elevated plasma volume. Hence, basal and CCK-induced oxytocin neuronal activity is expected to be attenuated.
2. Peripheral and central oxytocin release might be associated with, or be independent of each other (Wotjak et al., 1998; Sabatier et al., 2003a). However, systemic CCK administration results in peripheral as well as central release of oxytocin (Verbalis et al., 1986a; Renaud et al., 1987; Neumann et al., 1994). During pregnancy, to

maintain hyperphagia, CCK-induced central excitatory responses may be attenuated, which might be reflected in the peripheral response.

3. CCK activates the HPA axis (Kamilaris et al., 1992). But HPA axis responses to various stimuli are attenuated during pregnancy (Russell et al., 2008). CCK stimulates the SON oxytocin neurones by evoking NA release onto them (Onaka et al., 1995a). This NA release is sensitive to endogenous opioid peptides as they act presynaptically near or within the SON to block CCK-evoked NA release and thus oxytocin neuronal activation (Onaka et al., 1995a). A tonic endogenous μ-opioid inhibition of oxytocin neuronal activity emerges centrally towards the end of pregnancy and is exerted at the presynaptic opioid receptors on the NA-ergic nerve terminals in the SON (Douglas et al., 1995; Brown et al., 2000a). This opioid tone attenuates the IL-1β-induced oxytocin neuronal (secretory and electrophysiological) responses during pregnancy, and naloxone reverses this (Brunton et al., 2006). Hence, it is expected that CCK-induced excitatory electrophysiological responses of SON oxytocin neurones are also attenuated during pregnancy.
4. During pregnancy, the circulating level of leptin is elevated (Herrera et al., 2000). Although some hypothalamic regions become resistant to the actions of leptin (Ladyman and Grattan, 2004), present studies (Chapter IV - Leptin) show that SON oxytocin neurones are responsive to leptin during pregnancy. Hence, because of the elevated level of leptin, potentiation by leptin of CCK-induced responses might be expected during pregnancy in SON oxytocin neurones. However, it also appears that the CCK-NTS-SON pathway is not influenced by leptin in the normal non-pregnant state and hence there may not be any influence of pregnancy-induced elevation of leptin levels on the CCK-induced excitatory responses of SON oxytocin neurones.

Despite the above first three consensus, the CCK-induced oxytocin neuronal excitatory response in late pregnant rats was not different from the virgin rats (Douglas et al., 1995). However, the opioid antagonist, naloxone, potentiated the CCK-induced oxytocin response in late pregnant rats suggesting that oxytocin neurone responses to CCK are attenuated by endogenous opioid peptides during pregnancy (Douglas et al., 1994; Douglas et al., 1995). For comparison with fasted pregnant rats, CCK-induced responses of SON oxytocin neurones were also studied in unfasted pregnant rats in this study.

6.3.10. Comparison of CCK responses with responses to secretin and leptin

Secretin, the brain-gut peptide, and leptin, the anorexigenic peptide from adipose tissue, are also released postprandially (Schafmayer et al., 1978; Saladin et al., 1995). Secretin and CCK are physiologically similar peptides and both are synergistic in some of

the physiological functions (You et al., 1983; Chey et al., 1984). Leptin potentiates the peripheral and central actions of CCK (Barrachina et al., 1997; Wang et al., 1998; Emond et al., 1999; Matson and Ritter, 1999). Hence, CCK closely interacts with secretin and leptin. The present electrophysiological study shows that these peptides alter the electrical activity of SON neurones upon systemic administration. Hence, the electrophysiological responses of SON oxytocin and vasopressin neurones to systemic administration of secretin and leptin were compared to the responses to CCK in an attempt to understand the overall response of SON neurones during the postprandial period.

6.4. Methods

6.4.1. Experiment 1: Effect of systemic administration of CCK on electrical activity of SON oxytocin and vasopressin neurones in urethane-anaesthetized unfasted/fasted virgin/pregnant rats in vivo.

Urethane-anaesthetized unfasted virgin rats were fitted with an i.v. cannula on the day of the experiment. The SON and neural stalk were exposed via the ventral transpharyngeal surgery. The effects of CCK (25µg/kg; i.v) on oxytocin and vasopressin neurones were compiled from previous experiments on other peptides where CCK was used to differentiate these neurones.

Groups:

1. Oxytocin neurones
    a. Unfasted virgin: n = 45
    b. Unfasted pregnant: n = 14
    c. Fasted virgin: n = 6
    d. Fasted pregnant: n = 4
2. Vasopressin neurones: Unfasted virgin:
    i. Non-phasic: n = 30
    ii. Phasic: n = 7

6.4.2. Experiment 2: Effect of systemic administration of leptin on the CCK-induced excitatory responses of SON oxytocin neurones in unfasted virgin rats

Urethane-anaesthetized unfasted virgin rats were fitted with an i.v. cannula on the day of the experiment. The SON and neural stalk were exposed via the ventral transpharyngeal surgery and the SON neurones were identified and recorded. Once an oxytocin neurone was identified with an excitatory response to CCK (25µg/kg; i.v) leptin (100µg/rat; i.v) was injected; 20min after leptin, CCK was injected again to compare the excitatory responses to CCK of SON oxytocin neurones before and after leptin.

6.5. Results

6.5.1. Body weight

Fasting did not alter the body weight significantly in virgin or pregnant rats, while pregnancy contributed to significant increase in body weight whether they are fasted or unfasted (Fig. 6.1). There was no interaction between feeding and reproductive status with respect to body weight ($F_{1, 64}$=0.95, P=0.3, two-way ANOVA).

6.5.2. Basal firing rate

The basal firing rate of oxytocin neurones in unfasted pregnant rats was significantly higher than all the other groups (Fig. 6.2). There was a significant interaction between feeding and reproductive status with respect to the basal firing rate ($F_{1, 71}$=7.5, P=0.008, two-way ANOVA).

6.5.3. Effect of systemic administration of CCK on the firing rate of SON oxytocin neurones in unfasted virgin rats

Systemic administration of CCK increased the electrical activity of SON oxytocin neurones (e.g. Fig. 6.3). The mean change in firing rate 0-10min after CCK was significantly higher than the basal rate across 10min (n=45; Fig. 6.4). The incidence of spikes occurring between 0-0.5s was significantly increased 0-10min after CCK compared to the basal 10min period as it is seen in the interspike interval histogram (Fig. 6.5a, b). There was a positive shift in the hazard plot after CCK (Fig. 6.6) without a significant increase in the ratio of peak early (<0.07s) to mean late (0.2-0.3s) hazard after CCK (Fig. 6.7) suggesting that there is no change in the shape of the hazard after CCK administration.

6.5.4. Effect of systemic administration of CCK on the firing rate of SON oxytocin neurones: Influence of fasting and pregnancy

The influence of fasting and pregnancy on the CCK-induced excitation of SON oxytocin neurones was studied in fasted virgin rats (n=6) and unfasted (n=14) and fasted (n=10) pregnant rats.

- Fasted virgin: Pre *vs.* 0-10min post-CCK: P=0.06, paired t-test.
- Fasted virgin *vs.* unfasted virgin: P=0.4, t-test (Fig. 6.8).
- Unfasted pregnant: Pre *vs.* 0-10min post-CCK: P=0.005, paired t-test.
- Unfasted pregnant *vs.* unfasted virgin: P=0.012, t-test (0-2min after CCK); P=0.2, t-test (0-10min after CCK) (Fig. 6.9).
- Fasted pregnant: Pre *vs.* 0-10min post-CCK: P=0.002, paired t-test
- Fasted pregnant *vs.* fasted virgin: P=0.99, t-test (Fig. 6.10)
- Fasted pregnant *vs.* unfasted pregnant: P=0.9, t-test (Fig. 6.11).

Overall, there was no interaction between feeding and reproductive status ($F_{1, 65}$ =0.01, P=0.9, two-way ANOVA; Fig. 6.12).

6.5.5. Effect of systemic leptin administration on CCK-induced excitatory response of SON oxytocin neurones in virgin female rats

CCK (25µg/kg; i.v) was administered before and after leptin (100µg/rat; i.v) and the responses were compared (e.g. Fig. 6.13). The basal firing-rate of 2.9 ± 0.32 spikes/s was increased significantly 1.5min after CCK injection in SON oxytocin neurones (n=21). After leptin, a repeat injection of CCK significantly increased the firing rate of 3.03 ± 0.36 spikes/s to a similar extent. The rates returned to basal by 15min after CCK. There was no significant difference between these two basal rates. The average firing rate 0-10min after CCK was significantly higher than the respective basal rates both before and after leptin treatment. The CCK-induced excitatory responses before and after leptin were not different from each other (Fig. 6.14).

6.5.6. Comparison of CCK-induced response of SON oxytocin neurones to the responses induced by systemic administration of secretin and leptin: Unfasted virgin female rats

The electrophysiological responses of SON oxytocin neurones to secretin (0.1µg/rat; i.v; n=26) and leptin (100µg/rat; i.v; n=23) were studied and compared to the response after CCK (25µg/kg; i.v; n=45). The firing rate 0-10min after the administration of these peptides were significantly higher than during the respective basal period. The excitatory response 0-10min after the administration of each of the peptides differed significantly (Fig. 6.15).

Secretin was far more potent than CCK: the lesser number of moles of secretin injected (0.1µg or 0.33pmoles in 100µl saline or 33nM solution; MW: 3027.4) brought about more excitation than the 199-fold greater number of moles of CCK (7.5µg or 6.6nmoles of CCK in 150µl saline for a 300g rat or 43.77µM solution; MW: 1142.2) and 187-fold greater number of moles of leptin (100µg or 6.17nmoles in 100µl saline or 61.7µM solution; MW: 16.2kD). Hence, in comparison with CCK, leptin was the least effective while secretin was the most effective peptide in terms of excitation of SON oxytocin neurones upon systemic administration.

The change in firing rate for the peptides from the peak of excitation fitted well with exponential curves (Fig. 6.16). For an exponential decay process, the equation is:

$$R_{(t)} = R_0 e^{-\lambda t}$$

where $R_{(t)}$ is the rate at time t after the peak and R_0 is the rate at the peak of excitation. λ (lambda) is called the decay constant.

The equation obtained for secretin, CCK and leptin are: $R_{(t)} = 2.3\ e^{-0.1t}$, $R_{(t)} = 1.6\ e^{-0.2t}$ and $R_{(t)} = 0.5\ e^{-0.2t}$, respectively. The half-life ($t_{1/2}$) was calculated from the formula

$$t_{1/2} = \tau \times \log_n 2$$

where τ (tau) is $1/\lambda$.

The estimated half lives of the peptides from the literature are: secretin: 1.5-3min (de Smul et al., 1974; Lehnert et al., 1974; Tanaka and Katayama, 1984), CCK: 1–2 min (Hameed et al., 2009) and leptin: 9.4 ± 3.0 min (Zeng et al., 1997). The half lives calculated from the change in firing rate in this study are: secretin: 5.1min, CCK: 3.8min, and leptin: 3.3min.

The decay of the number of moles of the peptides (Fig. 6.17a, b, c and d) in the circulation was calculated from the formula

$$N_{(t)} = N_0\, 2^{-t/t1/2}$$

where N(t) is the number of moles at time t, N_0 is the initial number of moles in the circulation and $t_{1/2}$ is the half-life of the peptide.

6.5.7. Comparison of CCK-induced excitatory responses of SON oxytocin neurones to the responses induced by systemic administration of secretin and leptin: Fasted virgin female rats

The electrophysiological response of SON oxytocin neurones to secretin (0.1µg/rat; i.v; n=3) and leptin (100µg/rat; i.v; n=8) were studied in fasted rats and compared to the response after CCK (25µg/kg; i.v; n=6). The increase in firing rate 0-10min after the administration of secretin was significantly higher than during the basal period while the responses observed 0-10min after leptin and CCK administration were not significantly different from the respective basal firing rate. The excitatory response 0-10min after the administration of secretin differed significantly from the responses 0-10min after leptin and CCK (Fig. 6.18).

The secretin-, CCK- and leptin-induced responses in fasted rats did not differ from unfasted rats.

There was no interaction observed between feeding status and peptide administration ($F_{2, 105}$=0.7, P=0.5, two-way ANOVA). Fasting did not influence the response ($F_{1, 105}$=0.001, P=0.97, two-way ANOVA) while the responses were significantly different for the peptides ($F_{2, 105}$=11.6, P<0.001, two-way ANOVA).

6.5.8. Effect of CCK on non-phasic SON vasopressin neurones in unfasted virgin female rats

The effect of CCK (25µg/kg; i.v) was studied in non-phasic (n=30) and phasic (n=7) vasopressin neurones. Twenty three of 30 non-phasic vasopressin neurones were inhibited

(Fig. 6.19 and 6.20) while seven did not respond to CCK (Fig. 6.21). On average, the response observed 0-5min after CCK was significantly lower than the change in firing rate during the basal period (Fig. 6.22).

6.5.9. Effect of CCK on phasic SON vasopressin neurones in unfasted virgin female rats

All the phasic vasopressin neurones tested were inhibited by CCK (e.g. Fig. 6.23). The activity quotient was reduced significantly 5min after CCK (Fig. 6.24). In 4 of 6 neurones, there was a rebound excitation 5-10min after CCK following CCK-induced inhibition which resulted in a higher activity quotient than the basal. However, there were no significant changes in the mean interburst interval (Fig. 6.25) or frequency within bursts (Fig. 6.26) after CCK administration.

The interspike interval histogram before and after CCK administration was plotted from non-phasic and phasic vasopressin neurones (n=37; 30 non-phasic and 7 phasic vasopressin neurones; Fig. 6.27a). There was a decrease in the number of spikes occurring with the interspike interval of less than 0.3s following CCK. The single negative exponential curves fitting well with the distal tails of the histograms but leaving the intervals of less than 0.2s unfit characterise vasopressin neurones (Fig. 6.27b). There was no change in the shape of the hazard plot following CCK (Fig. 6.28).

6.5.10. Comparison of secretin-, CCK- and leptin-induced electrophysiological responses of non-phasic SON vasopressin neurones in unfasted virgin rats

Systemic administration of secretin, CCK or leptin resulted in mixed responses from the non-phasically firing SON vasopressin neurones. Secretin (0.1µg/rat; i.v; n=21) excited 14 neurones (66.7%), inhibited 6 neurones (28.6%) and did not affect the activity of 1 neurone (4.8%) of 21 neurones tested. CCK (25µg/kg; i.v; n=30) inhibited 23 (76.7%) and did not affect 7 of 30 neurones. Leptin (100µg/rat; i.v; n=11) excited 3 neurones, inhibited 4 and did not affect the activity of 4 of 11 neurones tested.

Overall, there were no significant changes in the firing rate 0-10min after administration of secretin and leptin compared to the rate during the respective basal periods. However, CCK significantly decreased the electrical activity 0-5min after its administration. The change in firing rate 0-5min after CCK was significantly different from that after secretin and leptin (Fig. 6.29).

6.5.11. Comparison of secretin-, CCK- and leptin-induced responses of phasic SON vasopressin neurones in unfasted virgin rats

The effects of secretin (0.1µg/rat; i.v) and leptin (100µg/rat; i.v) on the responses of phasic SON vasopressin neurones were compared to the inhibitory response to CCK (25µg/kg; i.v). All phasic vasopressin neurones were excited after secretin (n=11), while in

the leptin group, 6 of 8 neurones were inhibited while 2 of 8 were excited. Systemic CCK inhibited all 7 phasic vasopressin neurones tested.

The activity quotient was significantly increased by secretin and decreased by CCK while leptin did not have any effect (Fig. 6.30).

The mean interburst interval was significantly decreased by secretin and unaffected by CCK and leptin. The mean interburst interval during the basal period and 0-5min after administration of peptides did not differ between the groups (Fig. 6.31).

The frequency within bursts during the basal period and during 0-5min after administration of peptides was not different from each other within or between the groups (Fig. 6.32).

To summarise:

1. As shown in earlier studies (Hamamura et al., 1991; Douglas et al., 1995), the electrical activity of SON oxytocin neurones was significantly increased by systemic administration of CCK in unfasted virgin and pregnant rats, and the response in unfasted pregnant rats was not different from the virgin rats.
2. Fasting did not attenuate CCK-induced responses of SON oxytocin neurones in virgin and pregnant rats.
3. Exogenous administration of leptin did not potentiate CCK-induced excitation of SON oxytocin neurones in unfasted virgin rats.
4. Systemic administration of CCK either inhibited (77%) or did not affect (23%) the firing rate of SON vasopressin neurones, as shown previously (Renaud et al., 1987).
5. Secretin was more effective peptide than CCK or leptin in terms of excitation of SON oxytocin neurones in unfasted and fasted virgin rats. CCK significantly inhibited phasic and non-phasic vasopressin neurones but secretin significantly excited phasic vasopressin neurones in unfasted virgin rats.

6.6. Discussion

6.6.1. CCK-induced excitation of SON oxytocin neurones in unfasted virgin rats

Food intake is accompanied by fluid intake (i.e. prandial drinking) resulting in volume expansion (Franci et al., 1989) and oxytocin mediates ANP release and thus natriuresis after volume expansion (Verbalis et al., 1991; Haanwinckel et al., 1995). Hence, postprandial CCK-induced excitation of SON oxytocin neurones and the consequent peripheral release of oxytocin are important for the regulation of postprandial natriuresis.

From the electrophysiological recordings in this study, there was no change in the shape of the hazard plot and in the ratio of peak early to mean late hazard before and after CCK. These findings indicate that CCK-induced activation of oxytocin neurones involves

increased activity in the excitatory inputs to the SON rather than changes in the intrinsic properties of the neurones. This is in accordance with the findings that CCK-induced excitation of oxytocin neuronal activity and peripheral oxytocin secretion are due to the increased NA excitatory inputs from the NTS (Onaka et al., 1995a).

6.6.2. CCK-induced excitation of SON oxytocin neurones in pregnant rats

Unlike the earlier reports (Douglas et al., 1995; Brunton et al., 2006), the basal rate of oxytocin neurones was higher in unfasted pregnant rats in this study. The reason for this discrepancy is not known. Compared to virgin rats, the oxytocin neurones recorded from the late-pregnant rats displayed a delay in the increase in firing rate following CCK: the increase in firing rate 0-2min after CCK was significantly different between these groups. This could be due to the increase in the plasma volume during pregnancy essentially diluting the concentration of CCK and thus delaying the peak response or due to changes in the expression of CCK receptors in the vagal afferents, if any. The attenuation of CCK-induced oxytocin neuronal excitation by endogenous opioid peptides that emerges towards the end of pregnancy (Douglas et al., 1995) might also be responsible for this delayed response. However, in accordance with earlier reports (Douglas et al., 1995), the overall excitation (0-10min after CCK) in late pregnant rats was not different from virgin rats. The plasma oxytocin concentration during late pregnancy following systemic injection of CCK was also similar to that of virgin rats in other studies (Koehler et al., 1994; Douglas et al., 1995). As oxytocin mediates its natriuretic effects mainly via ANP (Haanwinckel et al., 1995) and ANP-induced natriuretic responses are attenuated in late pregnant rats (Masilamani et al., 1994), if CCK-induced oxytocin release regulates natriuresis, it is possible that the natriuretic responses to CCK through ANP are attenuated, even without alteration in CCK-induced oxytocin release.

6.6.3. CCK-induced excitation of SON oxytocin neurones in fasted rats

CCK is a short term satiety signal (Peikin, 1989) and CCK administration results in central (anorectic) and peripheral (natriuretic) oxytocin release (Neumann et al., 1994). Hence, homeostatic mechanisms during fasting might be expected to strive to restrain CCK-induced anorectic and natriuretic effects of oxytocin resulting in the attenuation of basal activity and CCK-induced SON oxytocin neurone responses in fasted virgin and pregnant rats. On the other hand, fasting, as a stressor, might also be expected to increase the basal and CCK-induced activity. However, fasting for 18h did not alter CCK-induced responses in virgin and pregnant rats.

6.6.4. Leptin and CCK interaction in the excitation of SON oxytocin neurones

Leptin has been shown to synergistically interact with CCK or potentiate the effects of CCK in suppressing food intake, reducing body weight and inducing Fos expression in the hypothalamus (Barrachina et al., 1997; Wang et al., 1998; Emond et al., 1999; Matson and Ritter, 1999; Matson et al., 2000; Wang et al., 2000; Matson et al., 2002). However, subthreshold doses of either CCK or leptin or both were used in these studies and thus it is not clear whether leptin influences CCK-mediated effects. In another study, when effective doses were used, leptin reduced the level of Fos expression induced by CCK in the SON and PVN (Caquineau and Leng, 2009). In the present study, the doses of leptin and CCK administered were effective in inducing a significant response in oxytocin neurones when given alone. However, there was no interaction observed in the excitation of SON oxytocin neurones between leptin and CCK. This is in accordance with the lack of change in CCK-induced oxytocin neuronal response following reduction in the endogenous level of leptin in fasted rats or increase in the level of leptin in pregnant rats. As systemic CCK acts via a vagus-NTS-SON pathway, lack of potentiation of CCK-induced excitation by leptin observed in this study suggests that leptin does not influence this pathway.

The other possibilities for the lack of interaction observed in the study are: Insufficient dose of exogenous leptin to potentiate the response and inadequate interval between leptin administration and observation of the CCK response.

The dose of leptin (100µg/rat; i.v) seemed sufficient because doses less than or similar to this dose have been effective for other leptin mediated effects (Matson et al., 1997; Merino et al., 2008). Although some leptin mediated effects can be rapid (Spanswick et al., 1997; Powis et al., 1998; Honda et al., 2002), the potentiation by leptin of the pathway under consideration might be a slow process requiring administration of leptin well before the measurement of the CCK response.

6.6.5. Comparison of secretin-, leptin- and CCK-induced responses

The excitatory effects on SON oxytocin neurones of secretin (0.1µg/rat; i.v) and of leptin (100µg/rat; i.v) were compared to that of CCK (25µg/kg; i.v). The secretin-induced response was significantly greater, and the leptin-induced excitatory response was significantly lesser than the CCK-induced response; the responses induced by all the peptides gradually subsided and the basal firing rate was reached by 15 min. The homogeneous excitatory responses of SON oxytocin neurones to secretin, CCK and leptin suggest that these peptides contribute to fluid homeostasis through postprandial oxytocin release.

A solution of 0.1µg/100µl (33pmoles/100µl or 33nM solution; MW 3027.4) secretin contains 133 times fewer molecules than 5µg/100µl (4.4nmoles/100µl or 44µM solution; MW 1142.2) solution of CCK and 187 times fewer molecules than 100µg/100µl (6.2 nmoles/100µl or 61.7µM; MW 16.2 kD) solution of leptin used in the study. Hence, the excitation induced by i.v. secretin is robust considering the dose used in the study.

The estimated circulating half-lives of the peptides in rats are: CCK: 1–2 min (Hameed et al., 2009), secretin: 1.5-3min (de Smul et al., 1974; Lehnert et al., 1974; Tanaka and Katayama, 1984) and leptin: 9.4 ± 3.0 min (Zeng et al., 1997) i.e. the half-life of CCK ≤ secretin < leptin. The basal plasma level of secretin is 5.5-6 pg/ml (1.8 pM) (Kawamura et al., 1991; Li et al., 1995), of leptin is ca. 2ng/ml (12.3mM) (Kawai et al., 1997) and of CCK-8 is 7.7pg/ml (6.7 pM) (Linden *et al.*, 1989a; Linden *et al.*, 1989b) i.e. the plasma level of secretin < CCK < leptin. Hence, it appears that secretin, though at a lower circulating concentration, is more potent in its effects on oxytocin neurones than CCK and leptin. The affinity of the receptors to their respective ligands and the effectiveness of the activation of vagal afferents in response to the receptor signalling might be different for these peptides resulting in differences in the dose-response characteristics. These differences might be important in the differential regulation of their shared physiological functions such as activation of the SON neurones.

6.6.6. CCK-induced inhibition of vasopressin neurones

The pathway leading to inhibition of SON vasopressin neurones by CCK is not clear. However, the CCK-induced inhibition of vasopressin neurones might be the result of regulation by central oxytocin and vasopressin release within the SON following systemic CCK. Central vasopressin auto-inhibits vasopressin neuronal activity via V_{1a} receptors (Ludwig and Leng, 2006); but it is not known whether systemic CCK results in central vasopressin release. On the contrary, systemic CCK results in central release of oxytocin (Neumann et al., 1994) but it is not clear whether central oxytocin suppresses vasopressin neuronal activity. However, retrodialysis of an oxytocin receptor antagonist onto the SON elevated plasma vasopressin secretion under basal conditions (Neumann et al., 2006) suggesting a role for central oxytocin in the regulation of vasopressin neuronal activity.

In another study (see Chapter V - Secretin), benoxathian, an α_1-adrenoceptor antagonist, blocked the basal activity of SON vasopressin neurones. Hence, it appears that the basal activity of the SON vasopressin neurones depends on the excitatory NA inputs via α_1-adrenoreceptors. SON vasopressin neurones receive excitatory NA projections from the A1 cell group of the VLM (Day, 1989; Day and Sibbald, 1989) that are not activated by CCK but by baroreceptor activation following reduction in blood pressure (Day, 1989).

However, SON application of NA, although it excited vasopressin neurones, also depressed their response to activation of the A1 input (Khanna et al., 1993). The latter effect was mimicked by the α_2-adrenoceptor agonist clonidine (Khanna et al., 1993). Hence, CCK-induced inhibition of vasopressin neurones might be the result of action of CCK-induced NA release in the vicinity of SON vasopressin neurones on the α_2-adrenoceptors on the presynaptic terminals of the axons from A1 cell groups, thus abolishing the tonic NA release and the basal activity.

Parvocellular vasopressinergic neurones in the PVN are activated during fever and central administration of vasopressin is antipyretic acting via V_1 receptors (Cooper et al., 1987; Naylor et al., 1987; Naylor et al., 1988; Wilkinson and Kasting, 1989; Landgraf et al., 1990; Wilkinson and Kasting, 1993). Peripheral vasopressin results in hypothermia i.e. decreases normal body temperature by suppressing thermogenesis (Shido et al., 1984). As systemic administration of CCK also lowers body temperature (Kapas et al., 1988; Kapas et al., 1989; Rezayat et al., 1999; Szelenyi, 2001; Szelenyi et al., 2004), the CCK-induced inhibition of vasopressin neurones might be a thermoregulatory mechanism by abolishing the antipyretic actions of central vasopressin (if CCK administration inhibits central vasopressin release) and hypothermic actions of peripheral vasopressin.

Leptin did not induce significant responses in SON vasopressin neurones. The responses to secretin were variable: either excitatory or inhibitory. However, the inhibitory or lack of responses to CCK of vasopressin neurones were consistent. Hence, it appears that SON vasopressin neurones are predominantly in an inhibited state under the influence of CCK during the postprandial period. Physiologically, depending upon the water intake during feeding and fluid balance during the postprandial period, vasopressin neuronal activity might be modulated by the peptides released postprandially: activated by secretin or inhibited/ unaffected by CCK.

6.7. Conclusion

Systemic CCK excited SON oxytocin neurones and either inhibited or did not affect vasopressin neurones. CCK-induced excitation of SON oxytocin neurones was not different in unfasted/fasted virgin/pregnant rats. Leptin did not influence the CCK-induced excitation of oxytocin neurones. Secretin-induced excitation was greater than the CCK-induced response while the leptin-induced response was the least. In conclusion, appetite regulating peptides that are released postprandially, such as secretin, leptin and CCK, target SON neurones. Overall, these peptides excite oxytocin neurones and inhibit vasopressin neurones which may regulate postprandial natriuresis and thus ensuring fluid homeostasis, the alteration of which is inevitable during food intake.

CHAPTER VII

DISCUSSION AND CONCLUSION

7.1. Neuropeptide Y

The NPY chapter (Chapter III) showed that the NPY-induced oxytocin release is centrally mediated. Brunton *et al.* have shown that central NPY induces Fos expression in the SON and increases peripheral oxytocin release (Brunton et al., 2006a). This study clearly showed that NPY increases the electrical activity of oxytocin neurones.

The physiological implications behind this oxytocin secretion by the neurohypophysis induced by central NPY are hypothetical as the regulation of SON oxytocin neurones by NPY neurones in the brainstem and ARC lacks evidence:

Firstly, though there is enough evidence that NPY is co-localized with NA neurones in the VLM (i.e. in the A1 cell group that sends projections to SON vasopressin neurones) (Sawchenko et al., 1985; Harfstrand et al., 1987), the evidence for NPY co-localization with NA in the NTS in particular (i.e. in the A2 cell group that sends projections to the SON oxytocin neurones) is not clear. There is evidence that NPY is present in the A2 cell group (Harfstrand et al., 1987; Simonian and Herbison, 1997), and that it is absent (Sawchenko et al., 1985). It is also not known whether the NA neurones that project to the SON in particular co-express NPY (Fig. 7.1). NPY negatively and positively modulates NA-induced excitation of SON vasopressin neurones via pre- and post-synaptic receptors, respectively (Khanna et al., 1993). It is not known whether there is a similar mechanism operating in the NPY-induced excitation of SON oxytocin neurones.

Secondly, though it is known that NTS-NA neurones are activated by food intake, gastric distension, stimulation of vagal afferents, gut peptides and stress (Monnikes et al., 1997; Willing and Berthoud, 1997; Rinaman et al., 1998; Conde et al., 1999; Krukoff et al., 1999; Ohiwa et al., 2006; Appleyard et al., 2007), it is not known whether these neurones are NPY neurones and whether these neurones project to the SON oxytocin neurones.

Thirdly, though there is an evidence for ARC projection to the SON (Leng et al., 1988), it is not known whether ARC-NPY neurones in particular project to SON oxytocin neurones (Fig. 7.1). In addition, it is not known whether NPY-induced effects on oxytocin neurones are direct or involve interneurones. Whether NPY modulates central release of oxytocin is also not known. Hence, to understand the physiological implications behind NPY induced oxytocin secretion, these gaps in the literature need to be filled.

The NPY chapter also produced a controversy: central NPY excited SON oxytocin neurones in the late pregnant anaesthetized rats (Fig. 3.12) in contrast to the suppressed oxytocin secretion and Fos activation observed in late pregnant conscious rats (Brunton et

al., 2006a). *In vivo* electrophysiology is an excellent tool to study the effects of drugs/peptides on the nervous system with all the synaptic inputs intact; however, anaesthesia is the limitation which cuts off sensory inputs that might modify the effect of the peptide under study. With urethane in particular, the disadvantage is that it alters body fluid homeostasis, preservation of which is likely to be crucial, especially in pregnant animals. To resolve the controversial issues that arose between conscious and pregnant animals, the complex procedure of electrophysiological studies in conscious animals (to preserve sensory inputs and other physiological functions such as food and water intake) may be attempted as changing the anaesthetic to one that does not interfere with physiological functions seems not possible.

Another interesting finding was the excitation of SON oxytocin neurones by systemic NPY (Fig. 3.15). NPY is released from NA nerve terminals upon sympathetic stimulation (Lundberg et al., 1987; Morris et al., 1987; Pernow, 1988). Recently it has been shown that peripheral NPY modulates adiposity and mediates stress-induced obesity (Kuo et al., 2007). The pathway and the importance of oxytocin neurones in NPY-mediated sympathetic effects and adiposity are yet to be explored.

7.2. Leptin

This chapter (Chapter IV) answered the question whether systemic leptin exerts any effect on SON oxytocin neurones. Interestingly, the answer was 'yes'. However, in turn, several questions arose out of this chapter: Will a physiological level of leptin have any effect on SON oxytocin neurones [100µg/kg/day subcutaneous infusion of leptin in starved rats results in physiological leptin level measured in control rats (Watanobe et al., 1999); hence though the dose of leptin (100µg/rat; i.v) used in the study is nearly physiological, the bolus intravenous injection may not resemble the slow physiological release of leptin from the adipose tissue]? What is the non-genomic mechanism behind the immediate effect of leptin on electrical activity? Why was the response not potentiated in fasted animals [fasting-induced low endogenous level of leptin was expected to augment the response to exogenous leptin because fasting decreases insulin level in the circulation (Szepesi et al., 1971) and reduction in the insulin level increases hypothalamic sensitivity to leptin (Rosenbaum et al., 2002; Lustig et al., 2004)]? Hypothalamic leptin resistance seems region-specific (Ladyman and Grattan, 2004). Is the finding that SON oxytocin neurones in late-pregnant rats respond to leptin suggest that SON is not resistant to leptin during late pregnancy or is it another controversy arising through use of anaesthetized rats? What is the physiological implication behind leptin-induced oxytocin release? If it is postprandial natriuresis, is leptin indispensable in postprandial oxytocin release? Does leptin induce central oxytocin release

from the dendrites of magnocellular oxytocin neurones [because leptin-induced reduction in food intake seems to be partly mediated via central oxytocin (Blevins et al., 2004)]? Obviously, more studies are required to answer these questions.

7.3. Secretin

This chapter (Chapter V) answered several interesting questions that have never been asked since the discovery of the hormone more than a hundred years ago (Bayliss and Starling, 1902). Secretin was found to be a powerful peripheral stimulus for oxytocin neuronal activity and peripheral oxytocin release at a dose that is almost physiological (Fig. 5.20 and 5.21). NA pathways were found to be involved in secretin-induced activation of SON oxytocin and vasopressin neurones (Fig. 5.31, 5.33 and 5.34). Secretin seems to induce dendritic oxytocin release from the SON but this warrants more observations. However, the physiological implications behind secretin-induced peripheral and central oxytocin release need to be explored further. It is not known whether secretin is more important than CCK in regulating postprandial oxytocin release or it is just another example for redundancy in a physiological system.

CCK-induced satiety may partly be mediated via central oxytocin release (Neumann et al., 1994). Though secretin seems to induce central oxytocin release and secretin is released postprandially, it has never been shown to act as a satiety peptide. The two studies that observed food intake following intraperitoneal secretin in rats report that secretin does not reduce food intake (Lorenz et al., 1979; Garlicki et al., 1990); however, intravenous secretin decreased food intake in sheep (Grovum, 1981). Hence, more studies are needed to explore the involvement of secretin in postprandial satiety and food intake. In addition, as secretin receptor or oxytocin knockout results in impaired social behaviour (Winslow and Insel, 2002; Nishijima et al., 2006), a secretin-oxytocin link in behavioural regulation needs to be investigated.

Another interesting study would be to explore the pathway involved in the secretin-induced excitation of vasopressin neurones: the direct projections from the NTS to the SON vasopressin neurones are relatively few and do not influence vasopressin neuronal activity (Day and Sibbald, 1989; Shioda et al., 1990); instead, NTS stimulation activates vasopressin neurones through an indirect relay projection via the A1 cell group (Day and Sibbald, 1989). This study (Fig. 5.33) showed that secretin-induced excitation of vasopressin neurones is mediated via a NA pathway – it is not known whether it is one of the A1 or A2 pathways or other indirect pathways. The projections from the A1 cell group to the SON vasopressin neurones are involved in blood pressure regulation – they are activated in response to low blood pressure, hypovolaemia or haemorrhage and are inhibited during baroreceptor

activation and hence either increasing and decreasing vasopressin neuronal activity and peripheral vasopressin release, respectively, to regulate effective circulating blood volume and blood pressure (Day and Sibbald, 1990; Day et al., 1992; Smith et al., 1995). However, secretin administration, even at high doses (0.75-3 µmol/kg; i.v), results in only a slight increase in blood pressure (Sitniewska and Wisniewska, 1999) and thus secretin-induced excitation of vasopressin neurones remains unexplained in terms of mechanism and physiological role.

7.4. Cholecystokinin

This chapter (Chapter VI) addressed whether there are any differences in the response of SON oxytocin neurones to systemic CCK in unfasted/fasted virgin/pregnant rats: no differences were observed between these groups.; Douglas *et al.,* have also reported that the response to CCK of SON oxytocin neurones during late pregnancy was similar to the virgin rats with augmentation by naloxone administration (Douglas et al 1995).

The interesting observation was that the basal activity in fasted virgin rats did not differ from unfasted virgin rats, in contrast to the expectation that fasting might either reduce or increase the basal activity of oxytocin neurones as a consequence of hydration status or stress response, respectively. It is not known whether fasting-induced alterations in fluid homeostasis (that might strive to restrict natriuresis and thus reduce oxytocin release) and fasting-induced stress response (which increases the release of oxytocin – a stress hormone) compensate each other and thus nullify changes in the basal activity of SON oxytocin neurones.

A controversy that was pointed out in this and other chapters that studied pregnant rats was that the basal firing-rate of SON oxytocin neurones was higher in unfasted pregnant rats (Fig. 6.2). This is in contrast to the expectation that oxytocin neuronal activity is suppressed during late pregnancy and also contrasted with the observation of reduced basal activity of oxytocin neurones in late pregnant in urethane-anaesthetized rats by Douglas *et al.* and Brunton *et al.* (Douglas et al., 1995; Brunton et al., 2006b). The reason for this discrepancy is not known.

The study on the influence of leptin on CCK-induced SON oxytocin neuronal excitatory responses showed that leptin did not potentiate the CCK response (Fig. 6.14). Though the literature reports potentiating, synergistic or subadditive[12] effects of leptin on

[12] Additive effect: An effect in which two substances used in combination produce a total effect the same as the sum of the individual effects (The American Heritage® Medical Dictionary, 2004 by Houghton Mifflin Company). Subadditive effect: the function for the sum of two elements always returns something less than or equal to the sum of the function's values at each element (www.wikipedia.org/wiki/Subadditivity)

CCK-mediated food intake, daily calorie intake, body weight regulation and Fos expression in the hypothalamus, except for Yuan et al (Yuan et al., 2000) who have used effective doses of CCK and leptin and reported subadditive effects, others have used subthreshold doses of either CCK or leptin or both (Barrachina et al., 1997; Wang et al., 1998; Emond et al., 1999; Matson and Ritter, 1999; Matson et al., 2000; Wang et al., 2000; Matson et al., 2002) and thus it is not clear whether leptin indeed exerts any effect on CCK-mediated effects. However, the lack of potentiation observed here in this study on the influence of leptin on CCK-induced excitation of oxytocin neurones was not conclusive: as it was said above, though the doses of CCK and leptin employed here are the effective doses in inducing an excitatory response in oxytocin neurones, they are supraphysiological [<0.12µg/kg/h and 100µg/kg/day are the physiological rates of release for CCK and leptin, respectively (Moriyoshi et al., 1991; Watanobe et al., 1999)], and hence may not be applicable for the physiological systems. As CCK is a within-meal satiety signal and exerts its effect immediately and leptin is a long-term satiety signal and requires longer time to exert its actions, leptin was given 20min before CCK in expectation of potentiating effects of leptin. However, the 20min duration between leptin and CCK injections might have not been sufficient for leptin to exert its effects in case slow genomic mechanisms are involved, or leptin and CCK should have been injected at the same time point as a means of showing synergistic effect of leptin in case rapid non-genomic mechanisms are involved; hence, the mechanism of leptin action in this particular pathway needs to be more fully understood to study CCK-leptin interaction in the excitation of SON oxytocin neurones.

7.5. Conclusion

It is tempting to hypothesize that NPY, leptin and secretin that are released during the postprandial period excite SON oxytocin neurones, similarly to CCK, resulting in peripheral oxytocin release so as to regulate postprandial natriuresis (Fig. 7.2). Central release induced by these peptides might contribute to negative regulation of feeding related behaviour.

As CCK, secretin and leptin are involved in regulating exocrine pancreatic secretion, it is possible that oxytocin release is an additional mechanism through which all these peptides regulate exocrine pancreatic secretion as oxytocin itself modulates exocrine pancreatic secretion via insulin and glucagons (Bjorkstrand et al., 1996; Ferrer et al., 2000). It is also possible that peripheral oxytocin is involved in the feedback regulation of leptin, secretin and CCK via oxytocin receptors in the adipose tissue, the gut and the enteric nervous system (Bonne and Cohen, 1975; Egan et al., 1990; Welch et al., 2009) (Fig. 7.3).

Regulation of natriuresis and exocrine pancreatic secretion are important requirements during the postprandial period. Natriuresis is essential for fluid homeostasis while exocrine pancreatic secretion and central oxytocin-induced anorexia/satiety are essential for energy homeostasis. Hence, it appears that these peptides target SON oxytocin neurones ensuring water and energy homeostasis (Fig. 7.4). This redundancy in the regulation of SON oxytocin neurones by appetite peptides signifies the importance of oxytocin in appetite regulation.

In conclusion (Fig. 7.5), this study has shown that

1. NPY, leptin, secretin and CCK excite SON oxytocin neurones (Fig. 3.6, 4.5, 5.2 and 6.4).
2. Secretin-induced excitatory responses were greater, even at lower molar doses, than responses to other peptides tested (CCK, leptin, NPY) (Fig. 6.15).
3. Secretin dose-dependently increased SON oxytocin neurone electrical activity and peripheral oxytocin release (Fig. 5.20 and 5.21) (and possibly SON somato-dendritic oxytocin release; Fig. 5.22b) in anaesthetized rats.
4. Intracerebroventricular infusion and microdialysis studies with benoxathian (α1 adrenergic antagonist) revealed that secretin-induced excitation of SON oxytocin and vasopressin neurones involves central excitatory noradrenergic pathways (Fig. 5.31, 5.33 and 5.34).
5. Fasting for 18h did not alter the excitation of SON oxytocin neurones induced by secretin, CCK or leptin (Fig. 6.18).
6. The pathway leading to excitation of oxytocin neurones by CCK was not influenced by prior leptin administration (Fig. 6.14).
7. SON oxytocin neurones were responsive to leptin during late pregnancy; i.e. leptin resistance was not seen in late pregnancy (Fig. 4.10).
8. NPY-induced excitation of oxytocin neurones was intact in anaesthetised late pregnant rats, contrasting with attenuated oxytocin secretory responses observed previously in conscious late pregnant rats (Fig. 3.13).
9. Systemic NPY excited SON oxytocin neurones (fig. 3.15).
10. Systemic CCK administration inhibited or did not affect SON vasopressin neurones, as shown by others (Leng et al., 1991) (Fig. 6.22 and 6.24), while leptin had no significant effect (Fig. 4.18 and 4.20), and responses to secretin were predominantly excitatory (Fig. 5.10 and 5.12). Systemic NPY resulted in a non-significant inhibition of vasopressin neurones (Fig. 3.20 and Fig. 3.22), but central NPY was ineffective (Fig. 3.16).

APPENDIX

1. Artificial cerebrospinal fluid (aCSF)

Solution A

8.1g/L Sodium chloride (138mM)

0.25g/L Potassium chloride (3.36mM)

0.8g/L Sodium bicarbonate (9.52mM)

0.07g/L Disodium hydrogen phosphate dihydrate (0.49mM)

0.13g/L Urea (2.16mM)

Solution B

0.14g/L Calcium chloride (1.26mM)

0.24g/L Magnesium chloride hexahydrate (1.18mM)

Solutions were made up with sterile double distilled water and stored at 4^{o}C until use. For use, solutions A and B were mixed at 9:1 ratio.

Final pH: 7.2

Osmolality: 295-300 mOsm

2. Reagents for plasma oxytocin radioimmunoassay

(1) Assay buffer

0.25g/L Sodium dihydrogen orthophosphate monohydrate

1.19g/L Disodium hydrogen orthophosphate anhydrous

1g/L Sodium azide (mM)

2.5g/L Bovine serum albumin

(2) Oxytocin standards

Standard oxytocin was obtained from the National Institute for Biological Standards and Controls (Hertfordshire, UK) as a freeze-dried aliquot containing 21µg of oxytocin. This was resuspended in assay buffer to give a 50ng/ml solution and stored in 1ml aliquots at -20^{o}C. This standard oxytocin aliquot was serially diluted to generate the following standards with a range of concentrations from 2.4 to 2500pg/ml.

A = 2500pg/ml 100µl of 50ng/ml stock + 1900µl assaybuffer

B = 1250pg/ml 500µl A + 500µl assay buffer

C = 625pg/ml 500µl B + 500µl assay buffer

D = 312pg/ml 500µl C + 500µl assay buffer

E = 156pg/ml 500µl D + 500µl assay buffer

F = 78pg/ml 500µl E + 500µl assay buffer

G = 39pg/ml 500µl F + 500µl assay buffer

H = 19.5pg/ml 500µl G + 500µl assay buffer

I = 9.7pg/ml 500µl H + 500µl assay buffer

J = 4.8pg/ml 500µl I + 500µl assay buffer

K = 2.4pg/ml 500µl J + 500µl assay buffer

(3) Assay control standards

The following oxytocin standards of 10, 50, 100, 250 and 500pg/ml were prepared in assay buffer, duplicate aliquots of which were incorporated at the beginning and end of the assay in order to assess the intra- and inter-assay variations. The remaining standards were stored at -20^{o}C for use in the subsequent assays.

IAS 0 = 1000pg/ml 400µl A + 600µl assay buffer

IAS 1 = 500pg/ml 500µl IAS 0 + 500µl assay buffer

IAS 2 = 250pg/ml 500µl IAS 1 + 500µl assay buffer

IAS 3 = 100pg/ml 500µl IAS 2 + 500µl assay buffer

IAS 4 = 50pg/ml 500µl IAS 3 + 500µl assay buffer

IAS 5 = 10pg/ml 500µl IAS 4 + 500µl assay buffer

(4) First antibody buffer

Normal rabbit serum obtained from Sigma (stored at -20^{o}C) was diluted 1:400 in assay buffer on the day of assay.

(5) First antioxytocin antibody

Rabbit antioxytocin antibody THF-3, donated by Takashi Higuchi (University of Fukui, Japan) was diluted 1:200 in assay buffer and stored at -20^{o}C. It was further diluted 1:1000 in first antibody buffer on the day of assay to give a final dilution of 1:200,000.

(6) Secondary antibody

Donkey anti-rabbit gammaglobulin (A-PPT1) was obtained from IDS Ltd (Boldon, UK) and stored at 4oC. On the day of assay, it was diluted 1:25 in assay buffer.

(7) Tracer: ^{125}I-oxytocin

370KBq of radioiodinated oxytocin (^{125}I-oxytocin) was obtained from Perkin Elmer Life and Analytical Sciences (Buckinghamshire, UK). It was diluted in 1ml of assay buffer and stored at -20^{o}C in 200µl aliquots. It is used at a concentration to give between 6000 and 8000cpm/50µl.

To calculate the volume of tracer required:

Total volume of tracer required = (Tube number of assay tubes X 0.05) + 2 = A ml

Activity required = 3.7KBq x A ml = B Kbq

If total volume of tracer available is C and current activity is D after decay, then the volume needed was: B/D x C. This was added to the required volume of buffer (A ml).

(8) Standardized Pansorbin cells

Pansorbin cells were obtained from Calbiochem Ltd (Nottingham, UK) as a 10% w/v solution containing 0.1% w/v sodium azide and stored at 4oC. This was diluted 1:25 in assay buffer on the day of use.

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LIST OF PUBLICATIONS

ABSTRACTS

1. **Velmurugan S,** Leng G and Russell JA (2009) Secretin, a brain-gut peptide, influences the electrical activity of supraoptic nucleus (SON) neurons via noradrenergic pathways in female rats. *Neuroscience 2009*, Chicago *(Accepted).*

2. **Velmurugan S,** Brunton, PJ, Leng G and Russell JA (2009) Secretin and leptin increase the electrical activity of supraoptic nucleus (SON) neurons in female rats: Comparison to CCK-induced excitation. *Neuropeptides – 19th Neuropharmacology Conference*, Chicago *(Accepted)*.

3. **Velmurugan S,** Brunton, PJ, Leng G and Russell JA (2009) Systemic secretin increases the electrical activity of supraoptic nucleus (SON) OT neurones and stimulates oxytocin (OT) secretion in the rat. *7th World Congress on Neurohypophyseal Hormones*, Kitakyushu, Japan **(Poster).**

4. **Velmurugan S,** Leng G and Russell JA (2009) Regulation of supraoptic nucleus (SON) oxytocin neurones by leptin and secretin in female rats *British Society of Neuroendocrinology Annual Meeting 2009*, University of Edinburgh, UK (**Poster**).

5. **Velmurugan S,** Leng G and Russell JA (2009) Secretin influences the electrical activity of supraoptic nucleus (SON) neurones in female rats via adrenergic pathways. *Annual Neuroscience Day meeting 2009*, University of Edinburgh, UK (**Poster**).

6. Russell JA, **Arunachalam S**, Brunton PJ (2008) Altered control of neurohypophysial hormones and blood osmolality and volume in pregnancy. *The 24th Congress of the Polish Physiological Society*, University of Life Sciences, Lublin, Poland.

7. **Arunachalam S**, Leng G and Russell JA (2008) Effect of systemic leptin administration on supraoptic nucleus oxytocin neurones: Influence of fasting and pregnancy in the rat. *British Society for Neuroendocrinology Annual Meeting 2008*, University of Bristol, UK (**Poster**).

8. **Arunachalam S**, Russell JA and Leng G (2008) Systemic leptin administration increases the electrical activity of supraoptic oxytocin neurones in urethane anaesthetized female rats. *The Young Physiologists Symposium and the Main Meeting of the Physiological Society*, University of Cambridge, UK (**Poster**).

9. **Arunachalam S**, Sabatier N, Brunton PJ, Leng G and Russell JA (2007) Centrally administered Neuropeptide Y (NPY) increases the firing rate of supraoptic oxytocin neurones. *World Congress on Neurohypophyseal Hormones*, Regensburg, Germany (**Poster**).

10. **Arunachalam S**, Sabatier N, Brunton PJ, Leng G and Russell JA (2007) Centrally administered Neuropeptide Y (NPY) increases the firing rate of supraoptic oxytocin neurons in urethane-anaesthetized late pregnant and non-pregnant rats. *British Society of Neuroendocrinology Annual Meeting 2007*, University of Nottingham, UK (**Oral**).

JOURNAL ARTICLES

1. **Sathya Velmurugan**, Paula J Brunton, Gareth Leng and John A Russell (2009) Systemic administration of secretin increases the electrical activity of supraoptic nucleus oxytocin and vasopressin neurons via noradrenergic pathways *(In Preparation)*

2. **Sathya Velmurugan**, Gareth Leng and John A Russell (2009) Systemic leptin increases the electrical activity of supraoptic nucleus oxytocin neurons in rats *(In Preparation)*

3. Paula J Brunton, **Sathya Arunachalam** and John A Russell (2008) Control of neurohypophysial hormone secretion, blood osmolality and volume in pregnancy. *Journal of Physiology and Pharmacology*, **59** (Suppl 8): 29-45.

4. Vicky Tobin, Philip M Bull, **Sathya Arunachalam,** Anne-Marie O'Carroll, Yoichi Ueta and Mike Ludwig (2008) The effects of apelin on the electrical activity of hypothalamic magnocellular vasopressin and oxytocin neurons and somatodendritic peptide release. *Endocrinology* **49** (12): 6136-45.

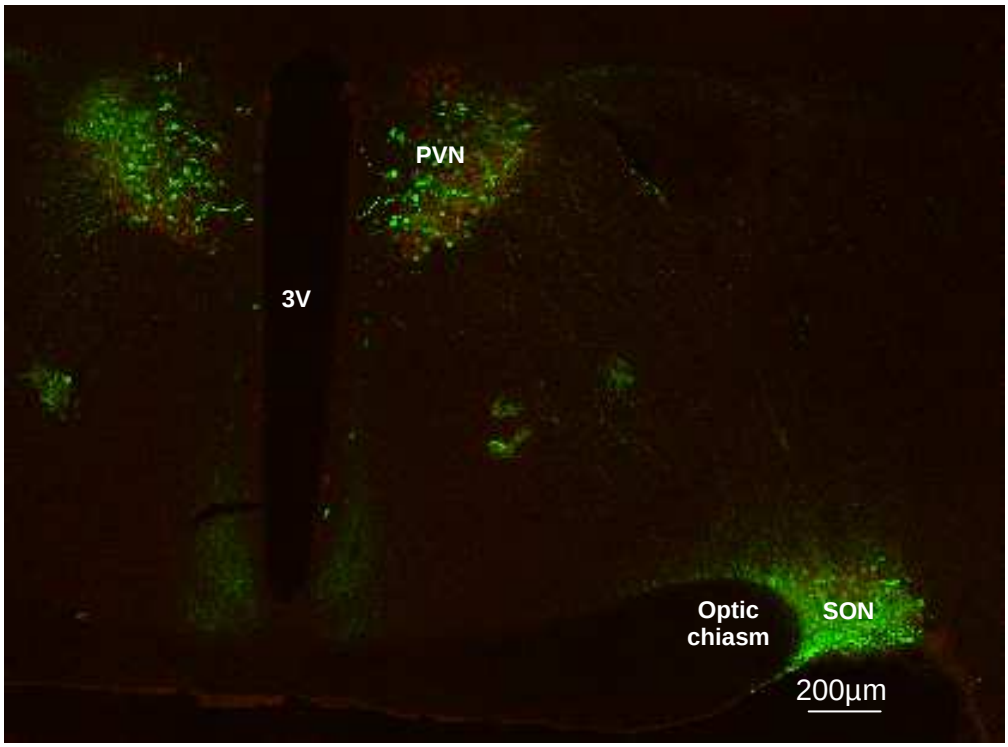

Fig. 1.1a. A coronal section of hypothalamus showing oxytocin system in the rat brain - Major sources of oxytocin: PVN and SON. Oxytocin neurones are stained with fluorescent red and vasopressin neurones with fluorescent green. Abbreviations: PVN, paraventricular nucleus; SON, supraoptic nucleus; 3V, third ventricle [From: Dr. Vicky Tobin, with permission].

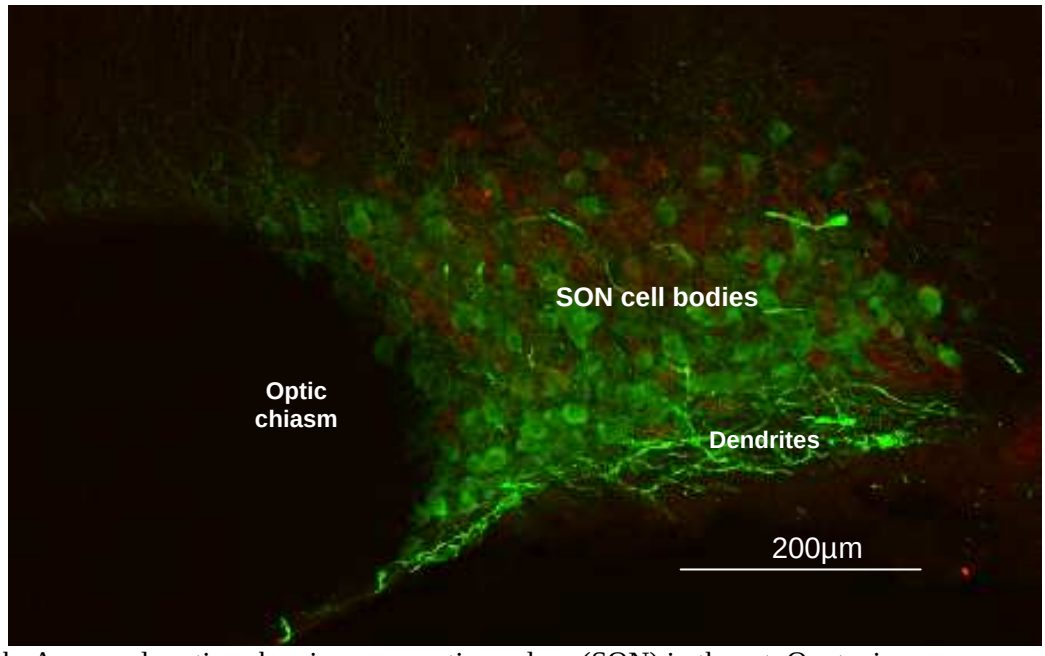

Fig. 1.1b. A coronal section showing supraoptic nucleus (SON) in the rat. Oxytocin neurones are stained with fluorescent red and vasopressin neurones with fluorescent green. The ventral dendritic lamina is one of the major sources of brain oxytocin [From: Dr. Vicky Tobin, with permission].

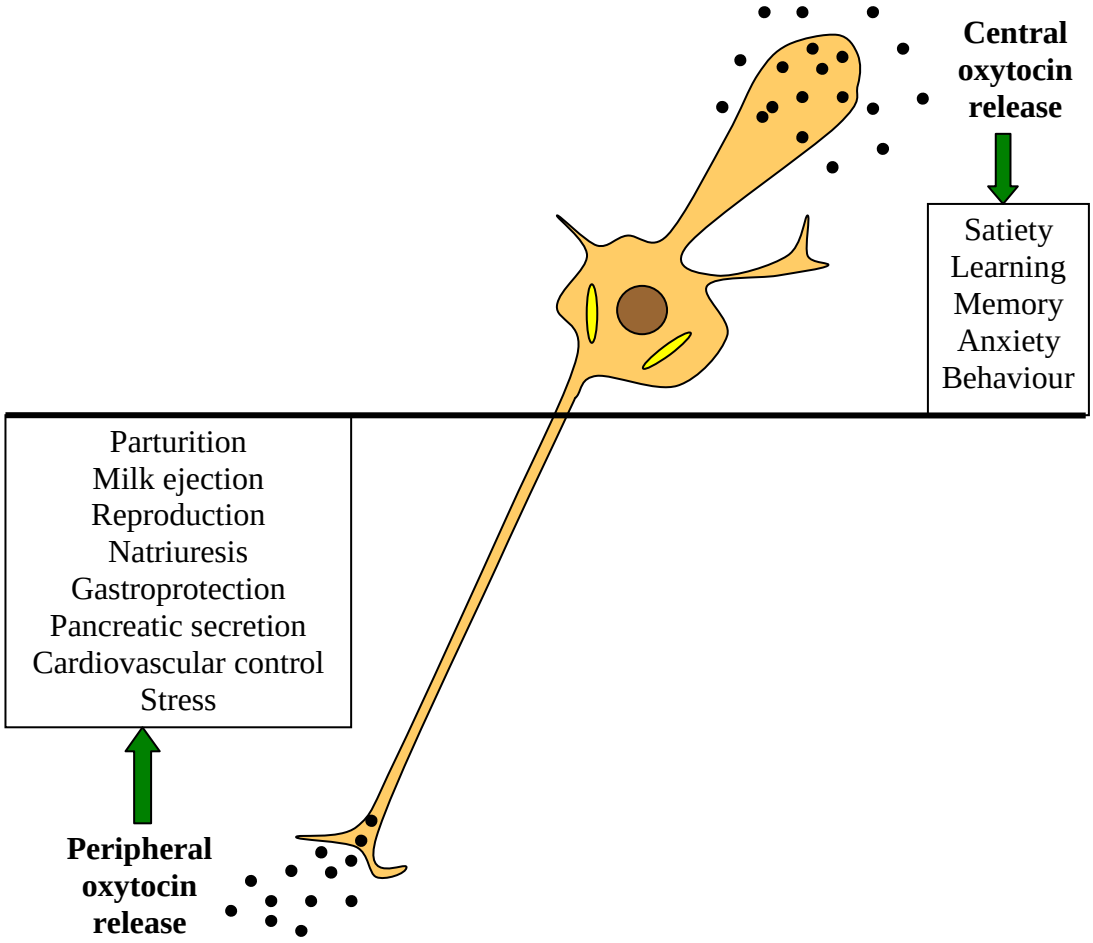

Fig. 1.2. An illustration of a magnocellular oxytocin neurone depicting regulation of various physiological processes by central and peripheral oxytocin. The role of peripheral oxytocin regulation of reproduction, fluid homeostasis, gastrointestinal system, cardiovascular system and stress has long been well established (Murphy, 1952; Chan and Sawyer, 1962; Nakano and Fisher, 1963; Fendler et al., 1964; Kaneto and Kosaka, 1970; Wakerley et al., 1973). The importance of central oxytocin in the control of reproductive and social behaviours, learning, memory and anxiety and stress was realised more recently (Engelmann et al., 2000; Heinrichs and Domes, 2008; Leng et al., 2008; Savaskan et al., 2008).

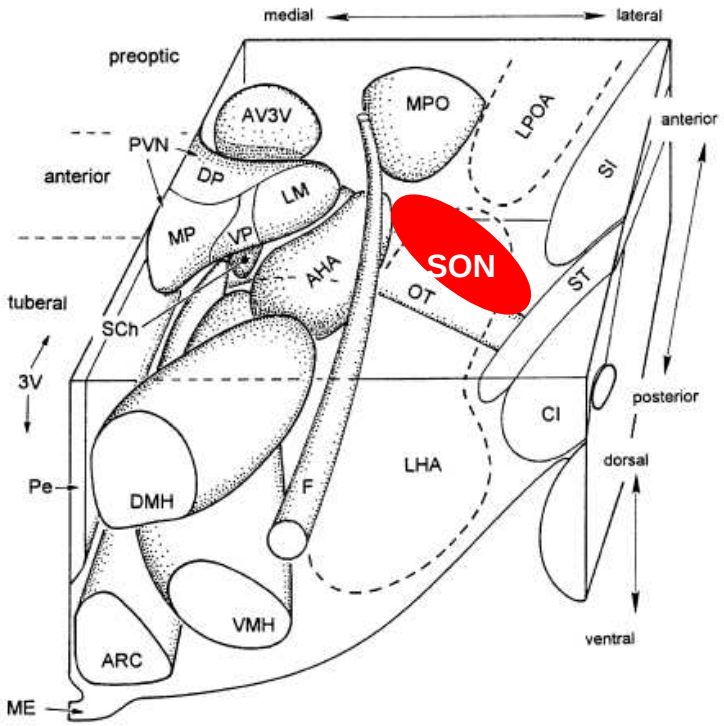

Fig. 1.3. Anatomical location of the SON in rat hypothalamus in relevance to the location of other appetite regulating centres: a 3D view from dorsal and caudal of major hypothalamic nuclei in the right hemisphere of rat hypothalamus. Abbreviations: AHA, anterior hypothalamic area; ARC, arcuate nucleus; AV3V, anteroventral area of third ventricle; CI, capsula interna; DP, dorsal parvocellular subnucleus of paraventricular nucleus (PVN); DMN, dorsomedial nucleus; F, fornix; LHA, lateral hypothalamic area; LM, lateral magnocellular subnucleus of paraventricular nucleus; LPOA, lateral preoptic area; ME, median eminence; MP, medial parvocellular PVN; MPO, medial preoptic area; OT, optic tract; SCh, suprachiasmatic nucleus; SON, supraoptic nucleus; SI, substantia inomminata; ST, subthalamic nucleus; VMN, ventromedial nucleus; VP, ventral parvocellular subnucleus of paraventricular nucleus. [Adapted from: Berthoud, 2002].

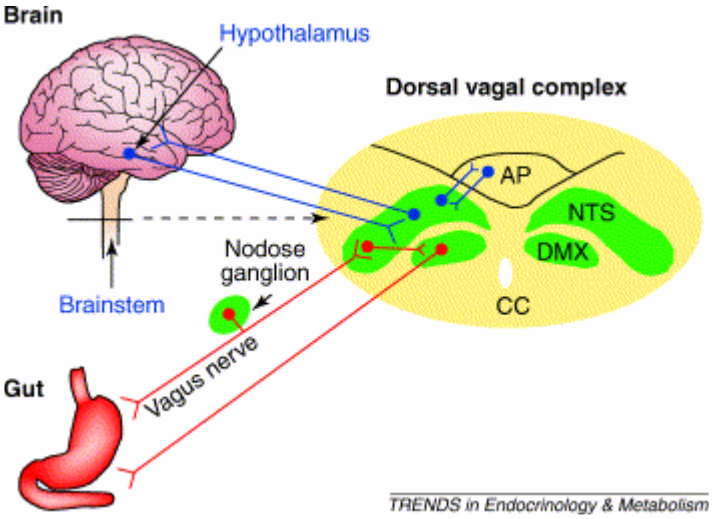

Fig. 1.4. Illustration showing the circuitry connecting abdominal vagal afferents, brainstem and hypothalamus. Connections in red show an autonomous reflex arc that forms a direct regulator of digestion. Connections in blue shows pathways required to integrate the reflex arc and the indirect regulators of feeding. Sensory afferents and motor efferents of the vagus serve as the connection between the gut and the dorsal vagal complex involves. Abbreviations: AP, area postrema; CC, central canal; DMX, dorsal motor nucleus of the tenth cranial nerve; NTS, nucleus of the tractus solitarius. [Adapted from: Luckman and Lawrence, 2003].

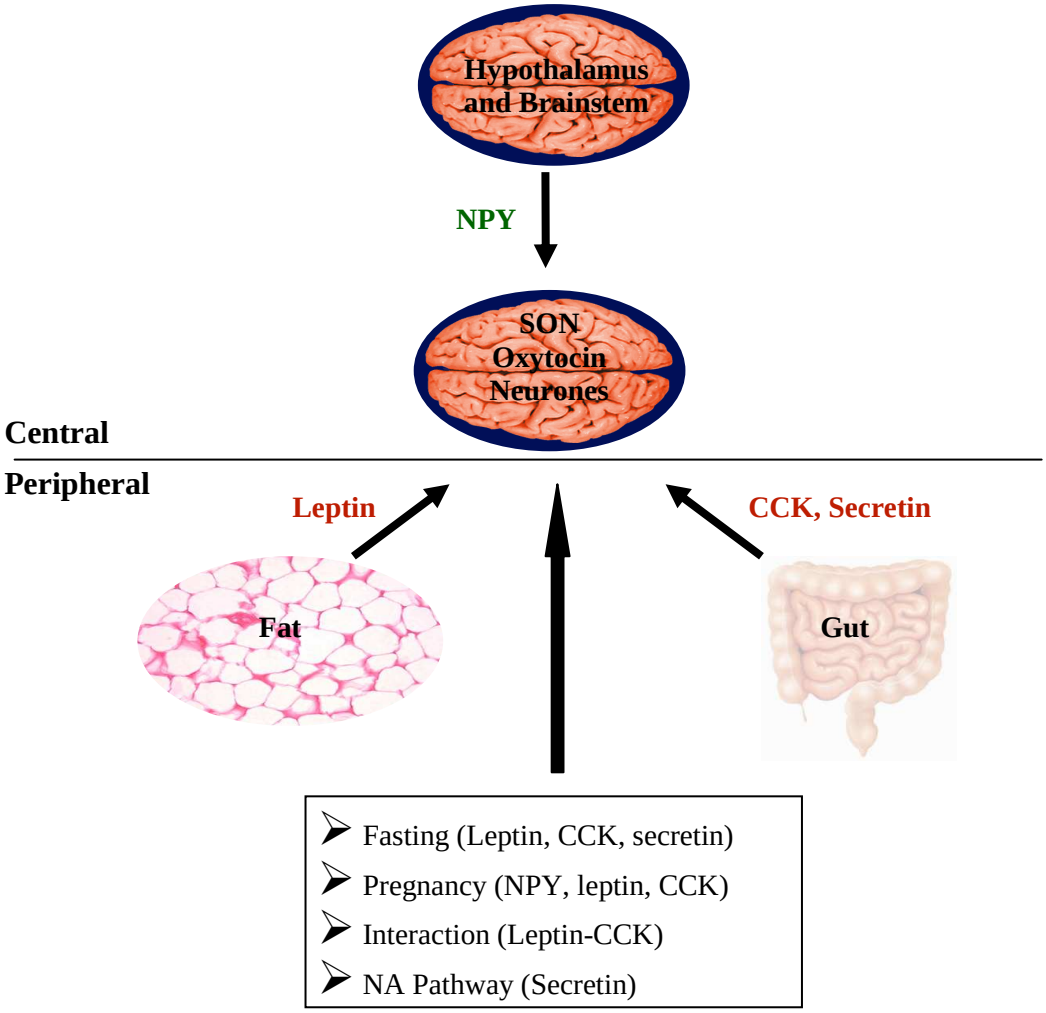

Fig. 1.5. Objectives of this thesis: To explore the responses of SON oxytocin neurones to four different appetite peptides of central and peripheral origin involved in stimulating or inhibiting appetite or in the signalling between the brain and the gut: neuropeptide Y (NPY, a central orexigenic peptide), leptin (a peripheral anorexigenic peptide from the adipose tissue), secretin (a brain-gut peptide from the gut) and cholecystokinin (CCK; a peripheral anorexigenic peptide from the gut). The influence of fasting and pregnancy on the effects on SON oxytocin neurones induced by the appetite peptides was also studied. The interaction between the long term anorectic peptide leptin and the short term anorectic peptide CCK was explored. The involvement of noradrenergic (NA) inputs on secretin-induced effects on SON oxytocin neurones was investigated.

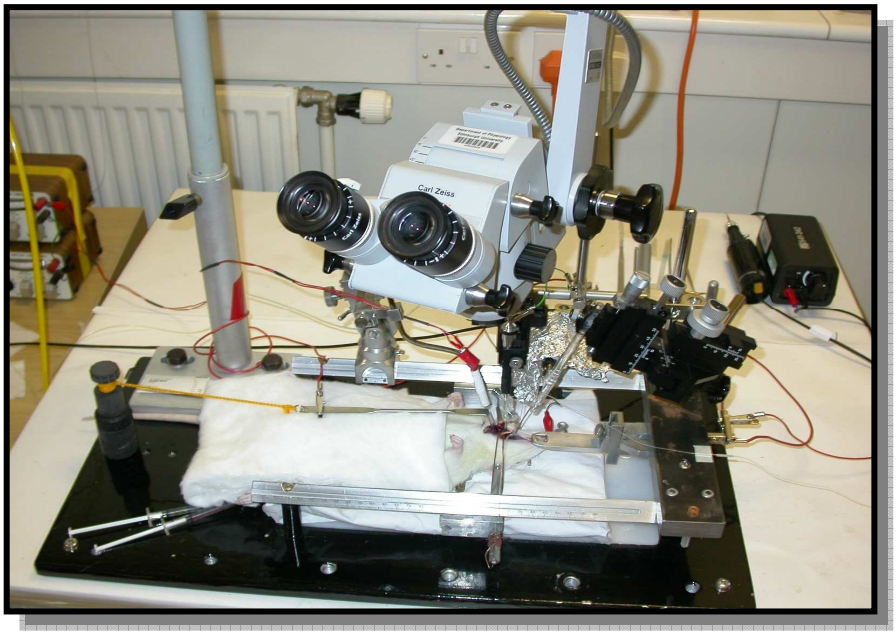

Fig. 2.1. *In vivo* electrophysiological experimental set-up: This illustration shows a urethane-anaesthetized rat positioned in a stereotaxic frame with the SON and neural stalk exposed through the ventral transpharyngeal surgical approach (Leng and Dyball, 1991). The stimulating and recording electrodes were held in place using stereotaxic holders. (Photograph from: Prof. Mike Ludwig, with permission).

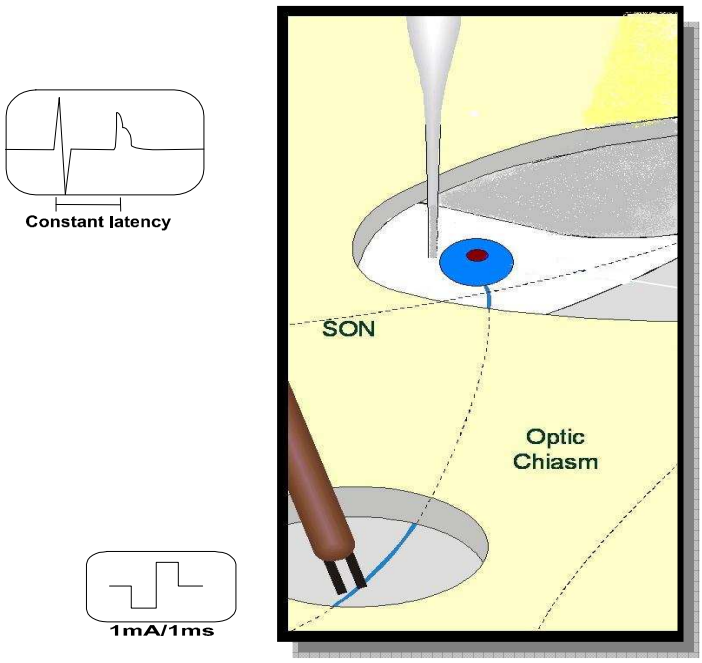

Fig. 2.2. Illustration showing the positioning of stimulating and recording electrodes in a rat model in which the SON and neural stalk were exposed through the ventral transpharyngeal approach. The stimulating electrode is positioned at the neural stalk to antidromically stimulate SON neurones with 1mA rectangular pulses for a duration of 1ms every 3s. This antidromic stimulation evokes an action potential at the cell body which is picked up by the recording electrode as a spike occurring at a constant latency following the stimulation artefact. [Illustration modified from: Ludwig and Leng, 1997].

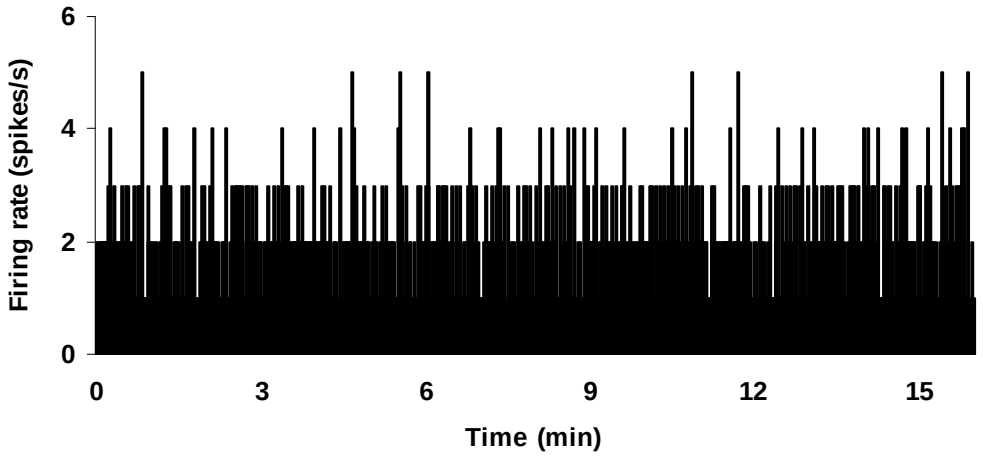

Fig. 2.3. Continuous firing pattern of a SON neurone (Cell No. 167-1). Mean +/- s.e.m. firing rate: 1.7 ± 0.05 spikes/s. This neurone is further classified as an oxytocin or vasopressin neurone depending upon the shape of the interspike interval histogram and hazard plot and the response to intravenous CCK.

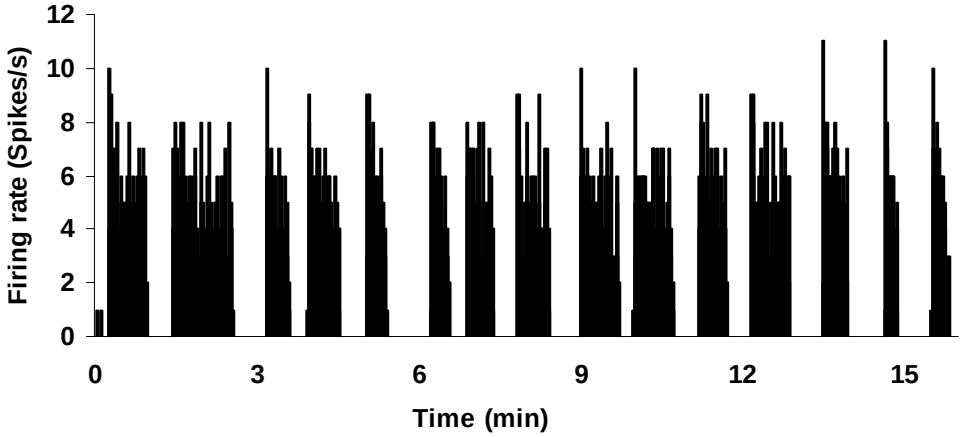

Fig. 2.4. Phasic firing pattern of a SON vasopressin neurone (Cell No. 119-1). Activity quotient: 0.1; frequency within bursts: 4.75spikes/s; mean interburst interval: 29.8s.

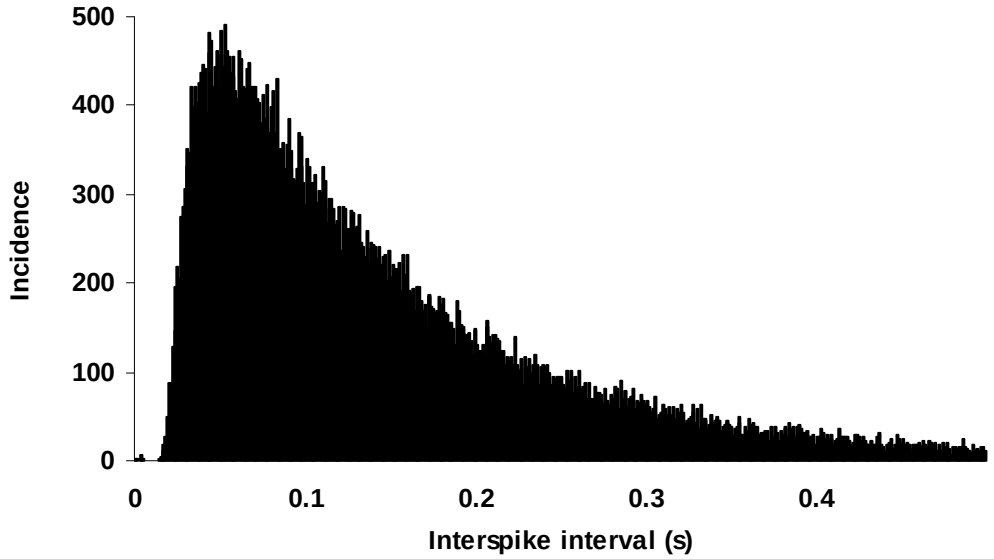

Fig. 2.5a. Interspike interval distribution of a single SON neurone (Cell No. 55-4). As the descending slope of the histogram fits perfectly with the single negative exponential curve (see below), this neurone was classified as a putative oxytocin neurone.

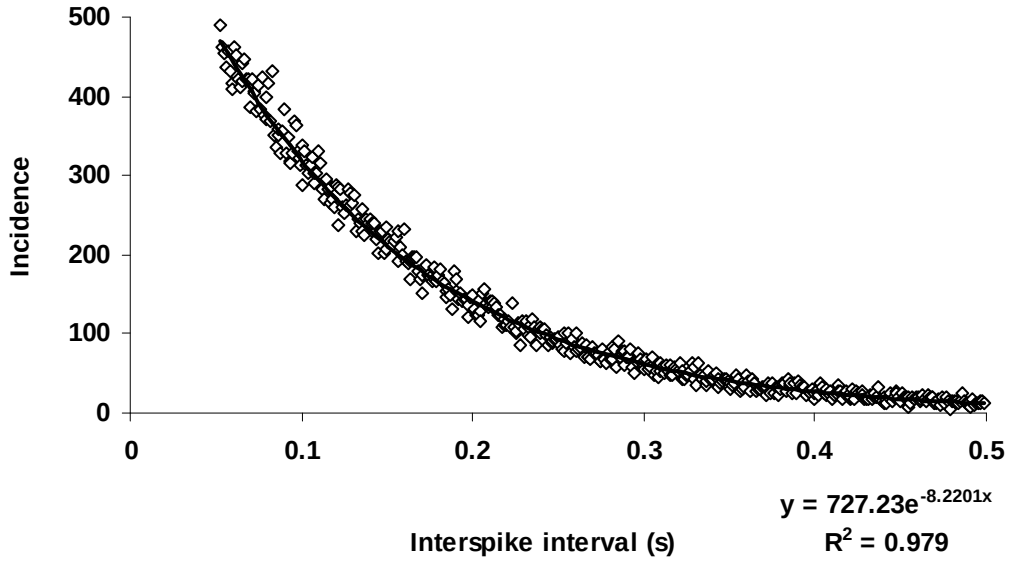

Fig. 2.5b. Interspike intervals of a single SON neurone (Cell No. 55-4) fitted to an exponential curve. The single negative exponential fits well with all data points (R^2 value of 0.98 indicates good fit), which is a characteristic of interspike interval histogram for SON oxytocin neurone. Hence, this neurone was classified as a putative oxytocin neurone.

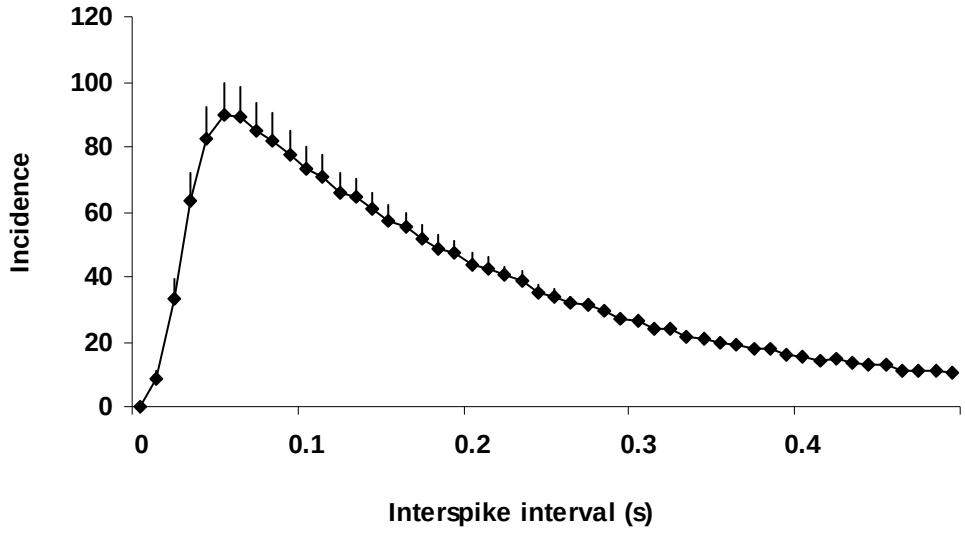

Fig. 2.6a. Interspike interval histogram constructed from 67 SON neurones identified as oxytocin neurones. Values are mean ± s.e.m; not normalised.

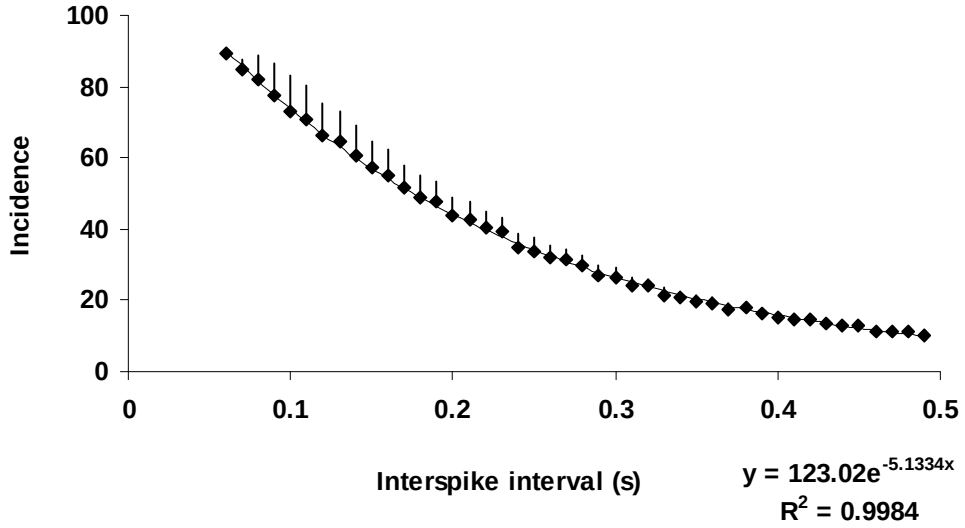

Fig. 2.6b. Interspike interval histogram constructed from 67 identified SON oxytocin neurones fitted to an exponential curve. Values are mean ± s.e.m. The single negative exponential fits well with all intervals which characterises SON oxytocin neurones. R^2 value of 0.998 indicates perfect fit.

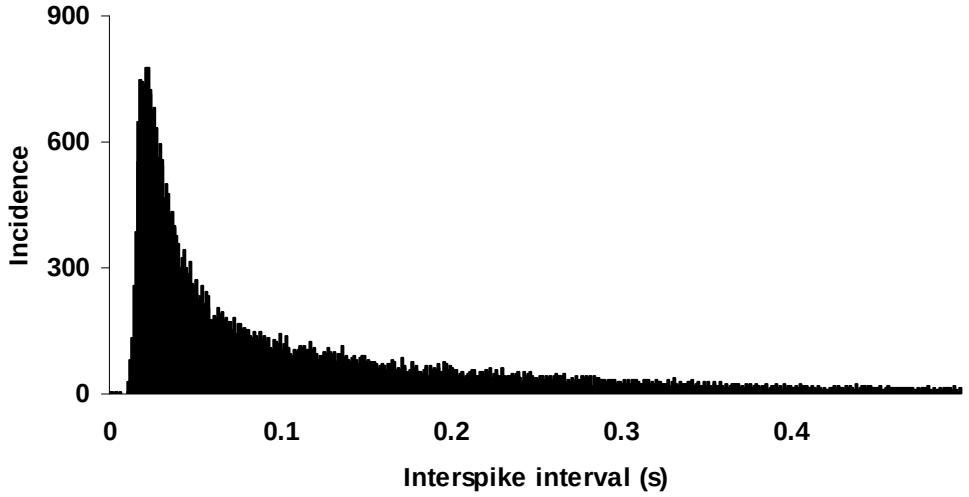

Fig. 2.7a. Interspike interval histogram of a single SON neurone (Cell No. 54-3). When fitted to a single negative exponential curve, the intervals less than 0.2s do not fit while the distal tail of the histogram fits well with the curve (see below). This is a characteristic of vasopressin neurones and hence, this neurone was classified as a putative vasopressin neurone.

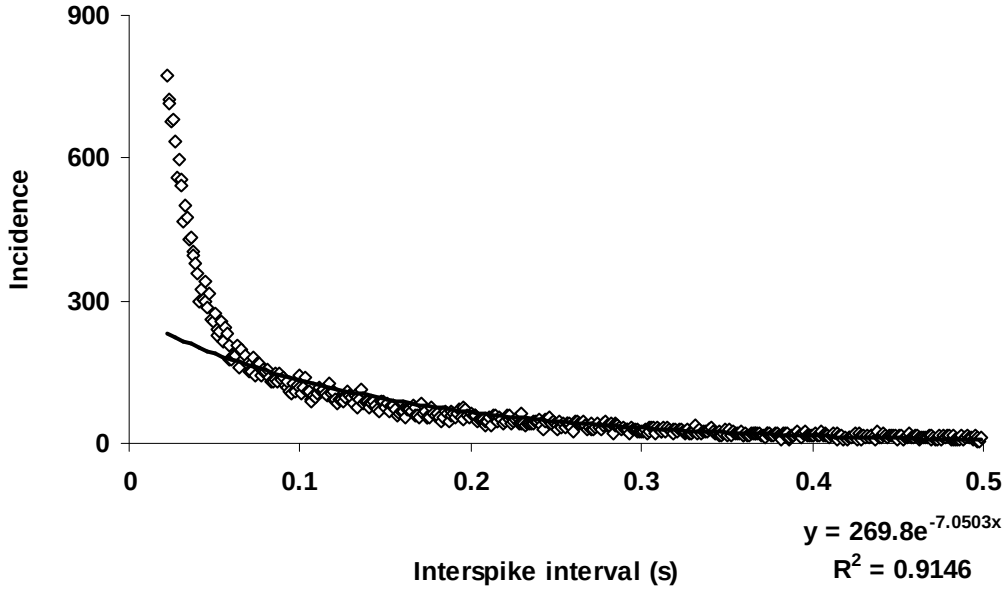

Fig. 2.7b. Interspike interval histogram of a single putative SON vasopressin neurone (Cell No. 54-3) fitted to a single negative exponential curve. The curve fits well with the distal tail of the histogram leaving behind the intervals less than 0.2s unfitted, which characterises this neurone as a SON vasopressin neurone.

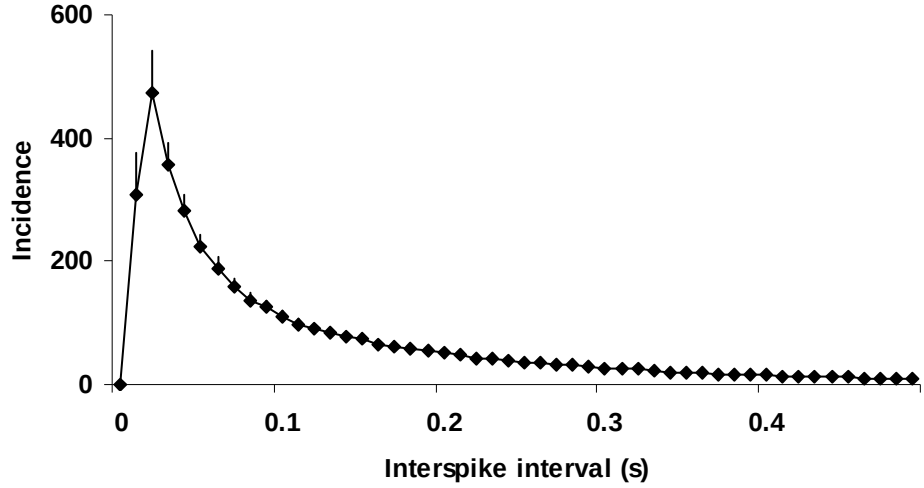

Fig. 2.8a. Interspike interval histogram constructed from 69 non-phasic SON neurones identified as vasopressin neurones. Values are mean ± s.e.m; not normalised.

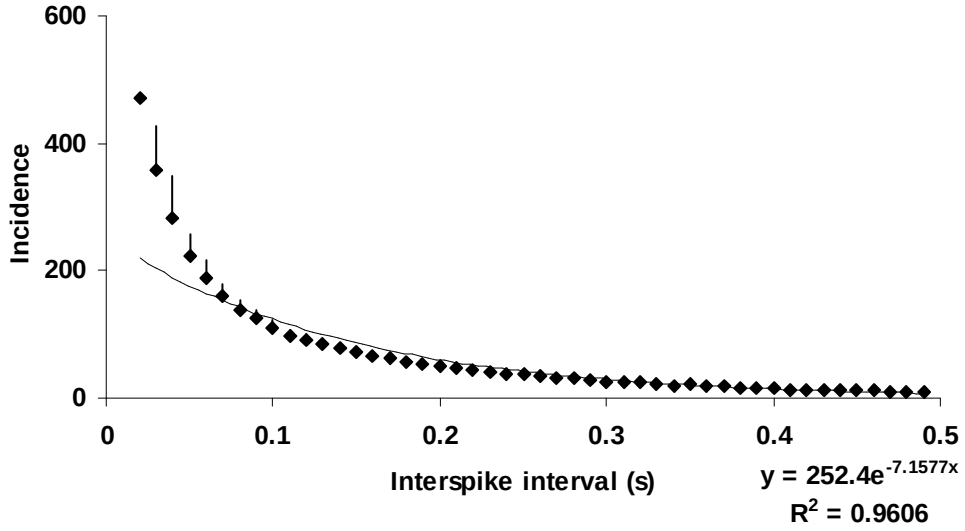

Fig. 2.8b. Interspike interval histogram constructed from 69 identified non-phasic SON vasopressin neurones fitted to a single negative exponential curve. Values are mean ± s.e.m. The curve fits well with the distal tail of the histogram leaving the intervals below 0.2s unfitted. This is a characteristic of histograms constructed from the firing of SON vasopressin neurones.

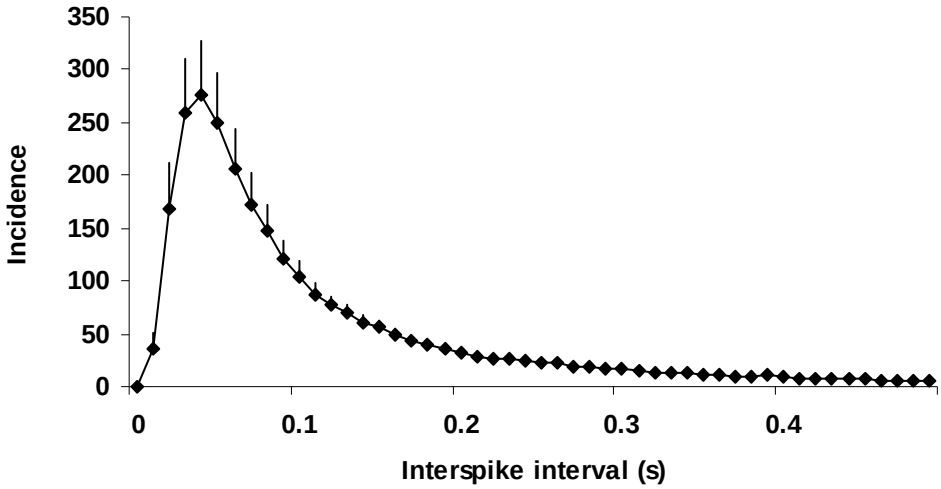

Fig. 2.9a. Interspike interval histogram constructed from 31 identified phasic SON vasopressin neurones. Values are mean ± s.e.m; not normalised.

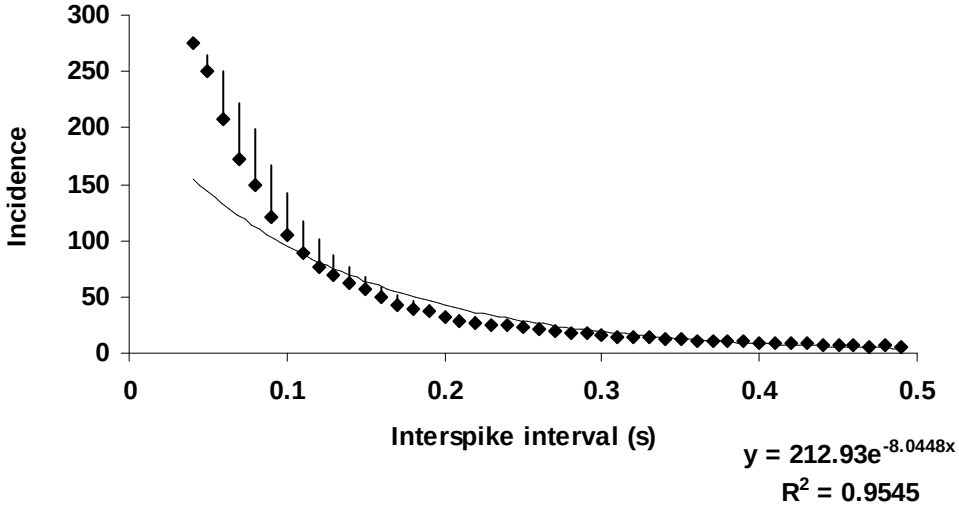

Fig. 2.9b. Interspike interval histogram constructed from 31 identified phasic SON vasopressin neurones fitted to a single negative exponential curve. Values are mean ± s.e.m. The curve fits well with the distal tail of the histogram leaving the intervals below 0.2s unfitted. This is a characteristic of histograms constructed from the firing of SON vasopressin neurones.

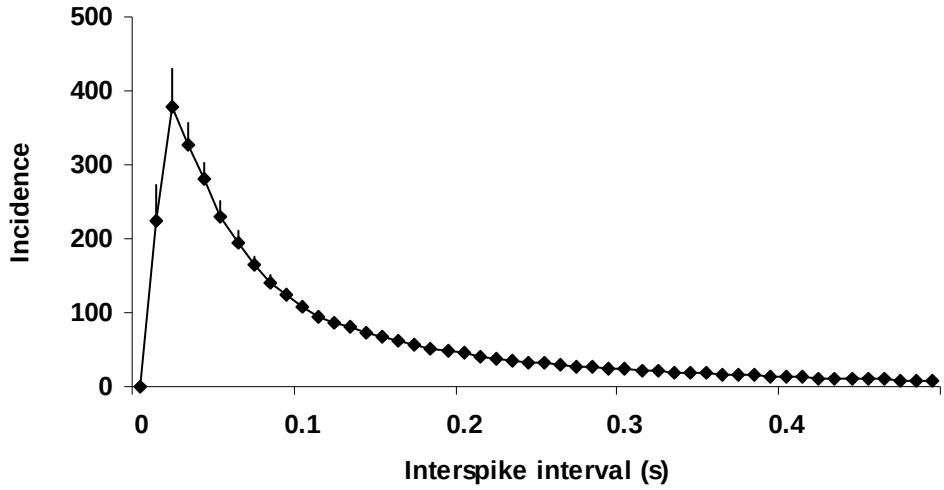

Fig. 2.10a. Interspike interval histogram constructed from 100 SON vasopressin neurones (69 non-phasic and 31 phasic cells combined). Values are mean ± s.e.m; not normalised.

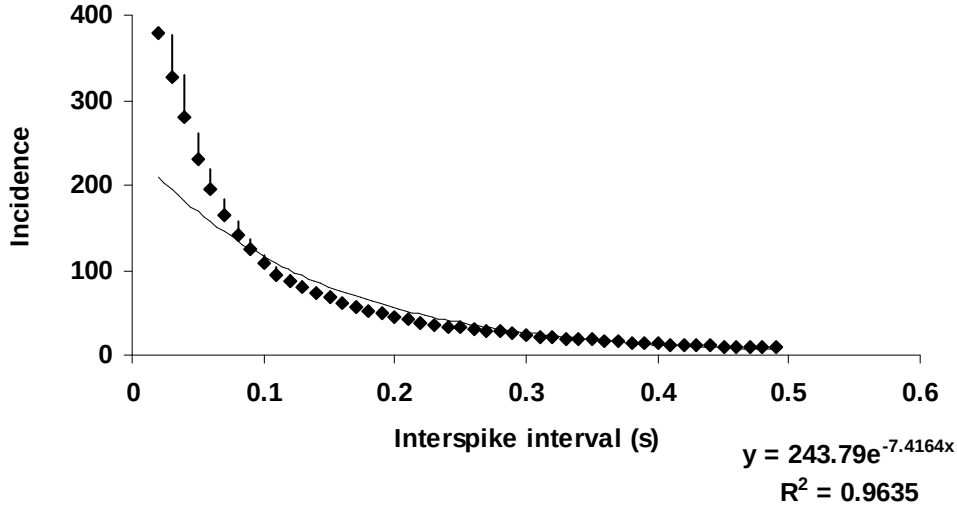

Fig. 2.10b. Interspike interval histogram constructed from 100 SON vasopressin neurones (69 non-phasic and 31 phasic cells combined) fitted to a single negative exponential curve. Values are mean ± s.e.m. The curve fits well with the distal tail of the histogram leaving the intervals below 0.2s unfitted. This is a characteristic of histograms constructed from the firing of SON vasopressin neurones.

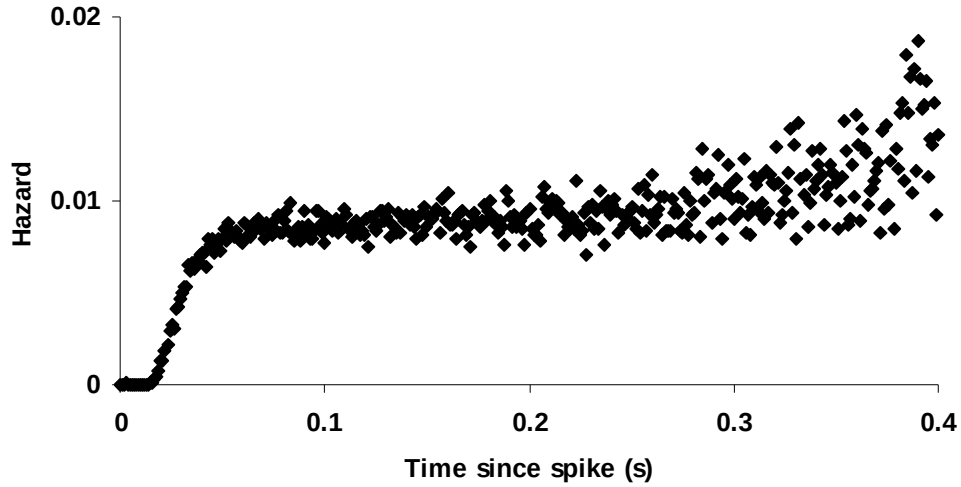

Fig. 2.11a. Hazard analysis of a single SON oxytocin neurone (Cell No. 55-4) classified as an oxytocin neurone based on the shape of the hazard plot. Hazard functions for oxytocin neurones show an initial refractory period, then a steady increase in hazard to a plateau after a post-spike interval of about 50ms, implying that after about 50ms, there are no detectable excitatory effects of an individual spike upon neuronal excitability (Sabatier et al., 2004).

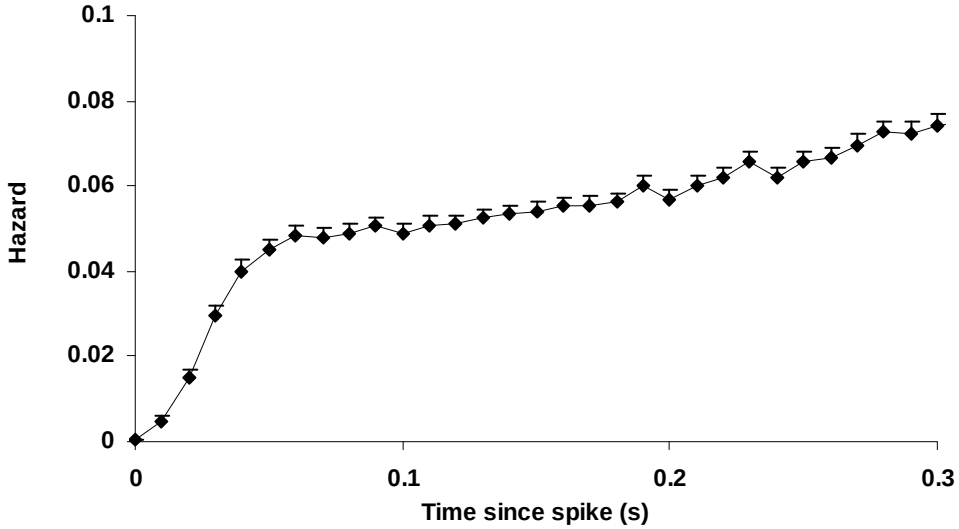

Fig. 2.11b. Hazard plot constructed from 67 SON neurones identified as oxytocin neurones. Values are mean ± s.e.m; not normalised.

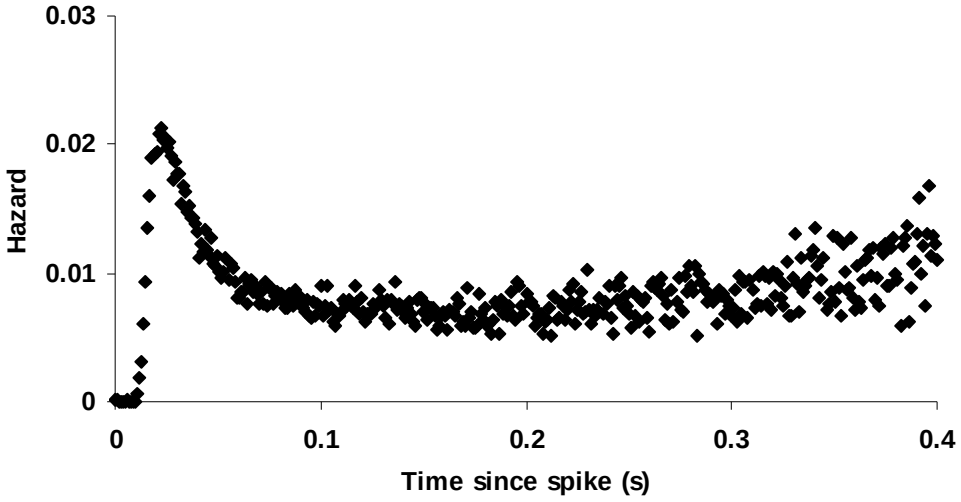

Fig. 2.12a. Hazard analysis of a single SON neurone (Cell No. 54-3) classified as a putative vasopressin neurone based on the shape of the hazard plot. Hazard analysis of vasopressin neurones show a low probability of discharge immediately after a spike, indicating a refractory period, followed by a peak of increased probability after each spike (from about 10 to 50 ms), which then decreases (Sabatier et al., 2004).

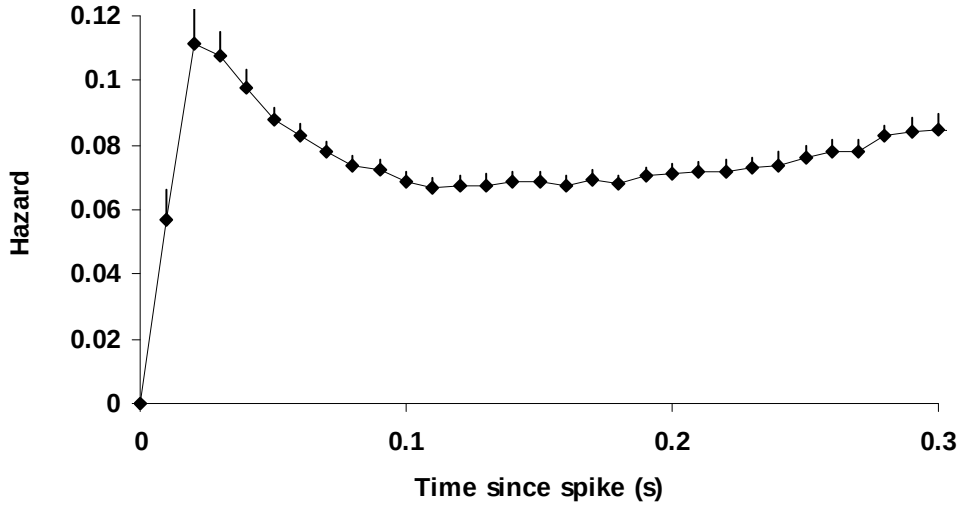

Fig. 2.12b. Hazard plot constructed from 69 non-phasic SON neurones identified as vasopressin neurones. Values are mean ± s.e.m; not normalised.

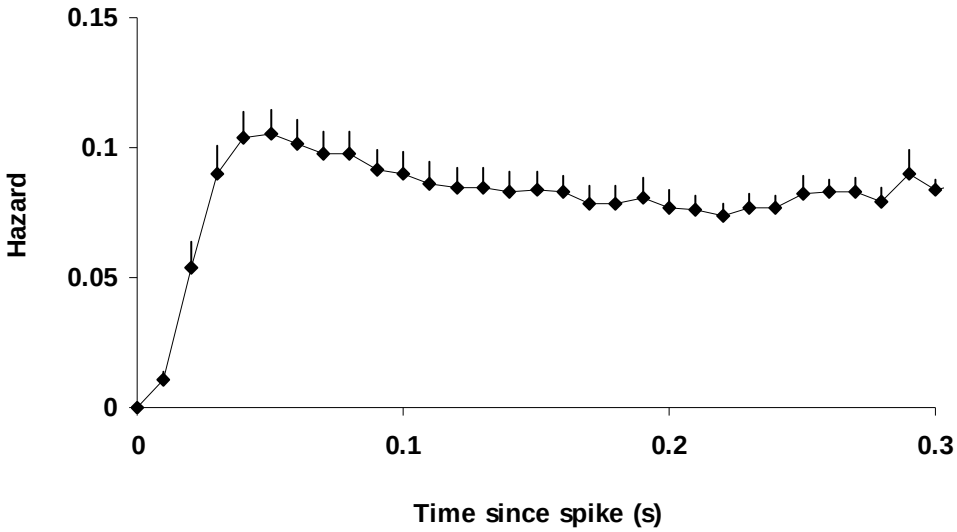

Fig. 2.12c. Hazard plot constructed from 31 phasic SON vasopressin neurones. Values are mean ± s.e.m; not normalised.

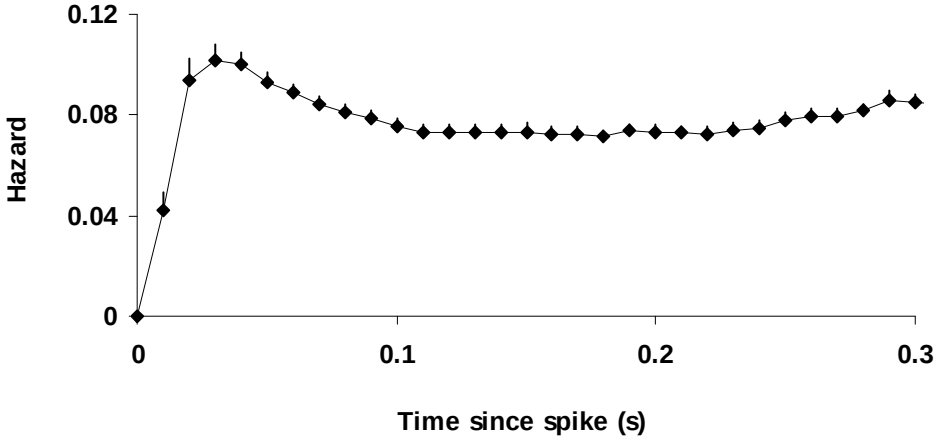

Fig. 2.12d. Hazard plot constructed from 100 SON vasopressin neurones (69 non-phasic and 31 phasic vasopressin neurones combined). Values are mean ± s.e.m; not normalised.

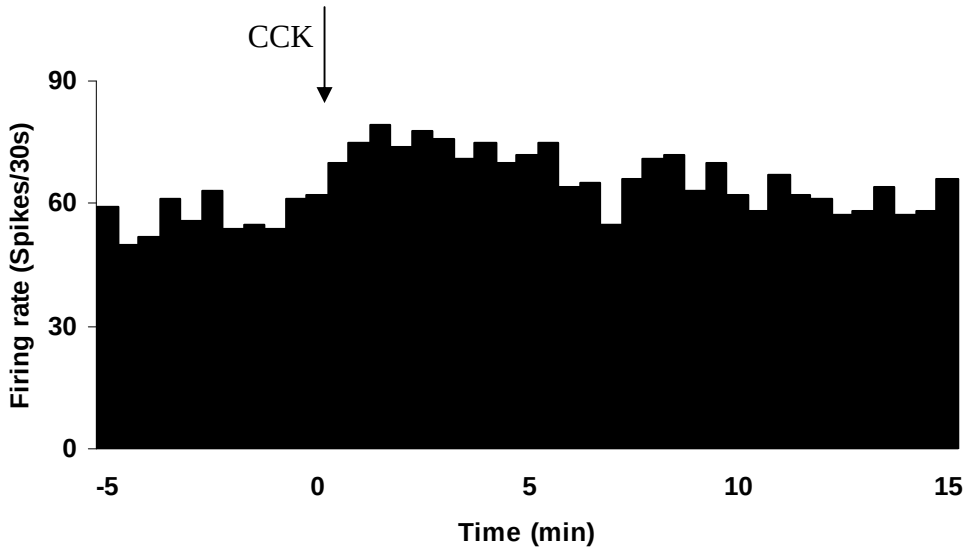

Fig. 2.13. Differentiation of oxytocin and vasopressin neurones by systemic CCK administration: The basal firing rate of 1.9spikes/s was increased by 2.6spikes/s (140% increase) 1.5min after administration of CCK (25µg/kg; i.v), which is a characteristic of oxytocin neurones. Hence, this neurone was classified as a SON oxytocin neurone (Cell No. 166-1).

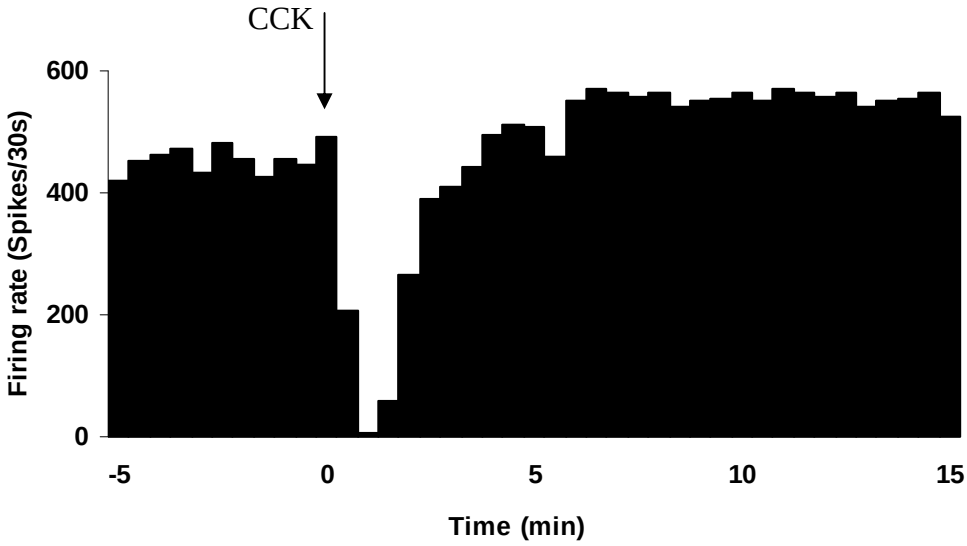

Fig. 2.14. Differentiation of oxytocin and vasopressin neurones by systemic CCK administration: The basal firing rate of 15spikes/s was decreased by 14.9spikes/s (99% decrease) 1min after administration of CCK (25µg/kg; i.v) which is a characteristic of vasopressin neurones. Hence, this neurone was classified as a SON vasopressin neurone (Cell No. 19-3).

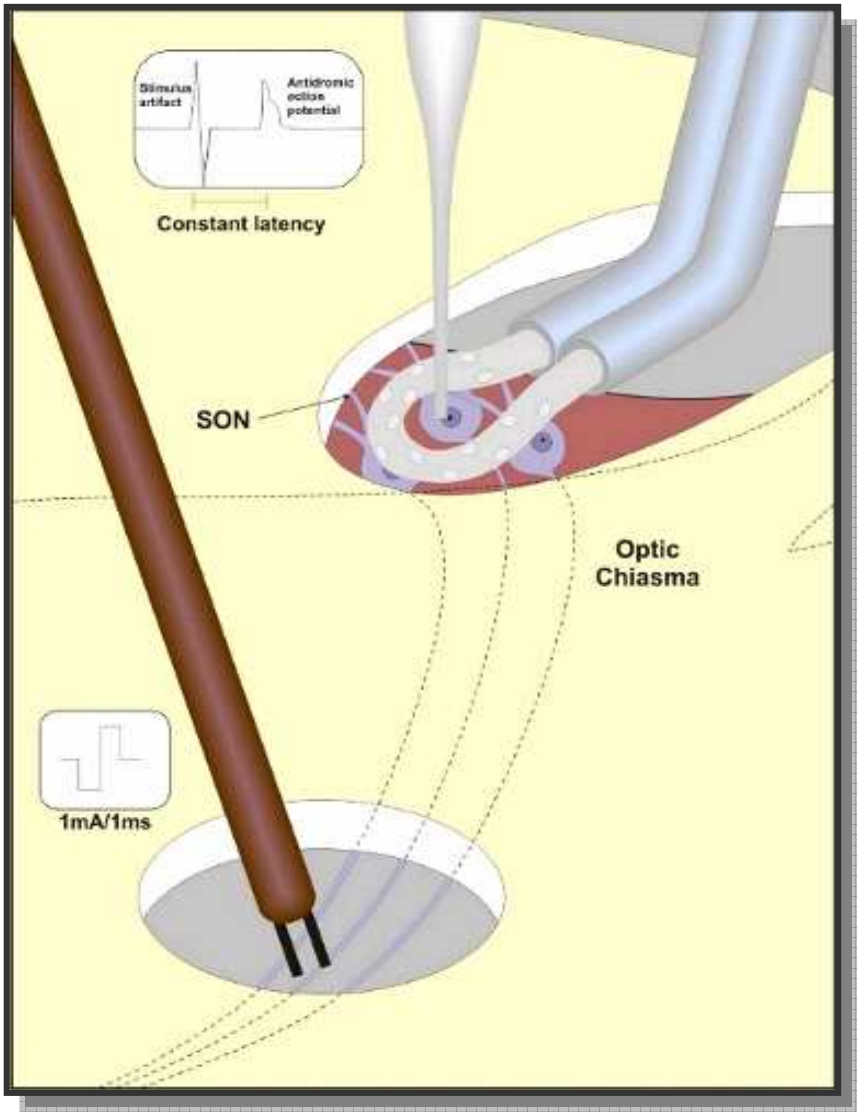

Fig. 2.15. This illustration shows the placement of a microdialysis probe in an *in vivo* electrophysiological set-up that allows simultaneous recording of firing rate of a SON neurone while a drug is microdialysed to study the local effect of the drug. This set-up was also used to collect the microdialysate to evaluate dendritic release of oxytocin in response to administration of drugs. [Adapted from: Ludwig and Leng, 1997].

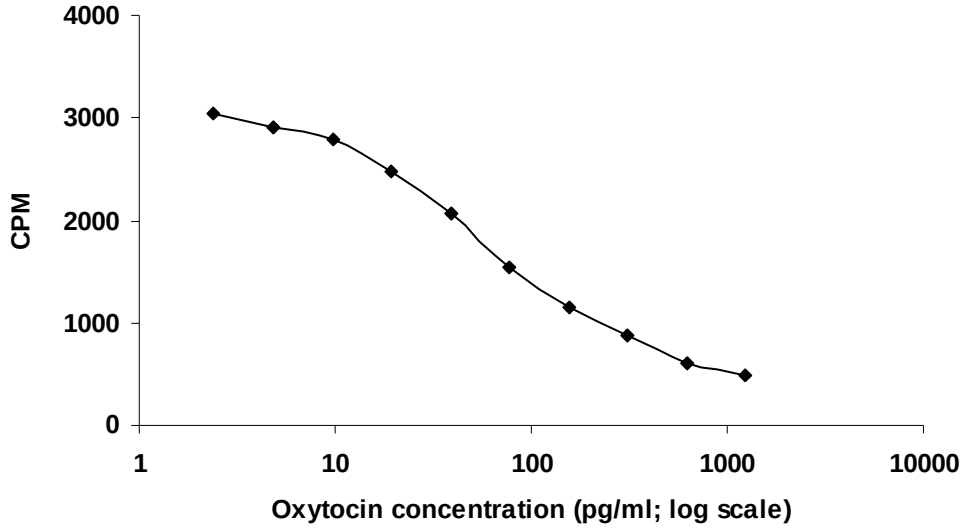

Fig. 2.16. Standard curve obtained for the measurement of plasma oxytocin concentration by radioimmunoassay (Assay performed with Dr. Paula J Brunton).

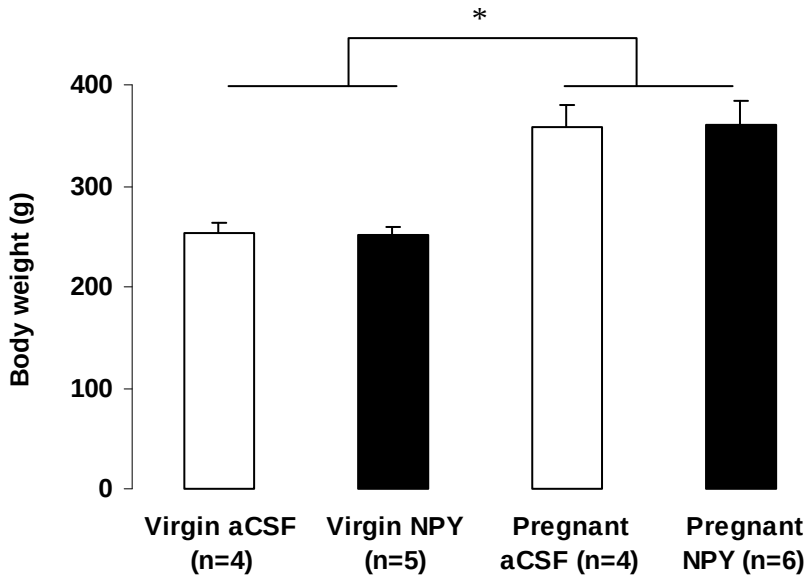

Fig. 3.1. Average body weights of virgin and pregnant rats used in the NPY study. Values are mean ± s.e.m. * P<0.001, $F_{1, 15}$=18.6, two-way ANOVA, virgin *vs.* pregnant groups.

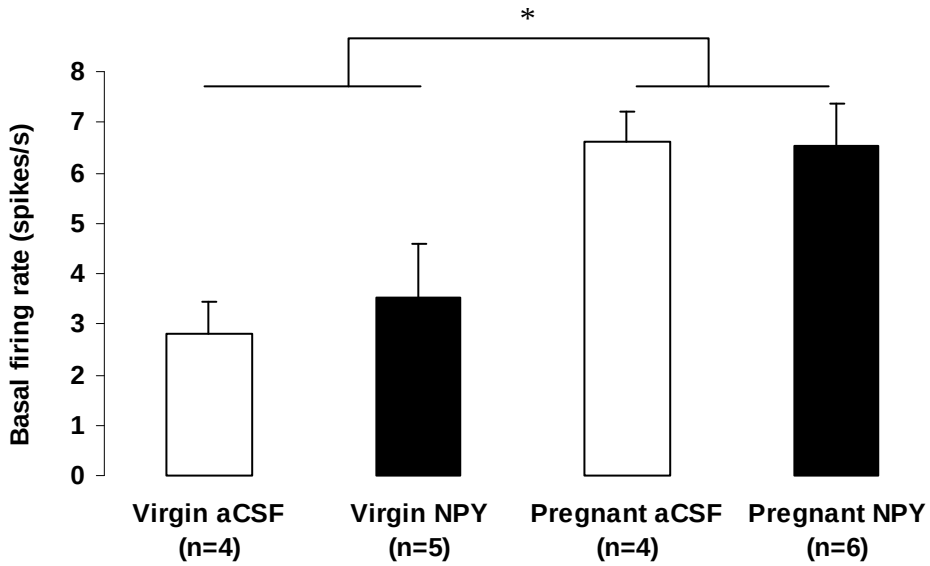

Fig. 3.2. The average basal firing rate of SON oxytocin neurones in the NPY study. Values are mean ± s.e.m. * P<0.001, $F_{1, 15}$=43.4, two-way ANOVA, virgin *vs.* pregnant groups.

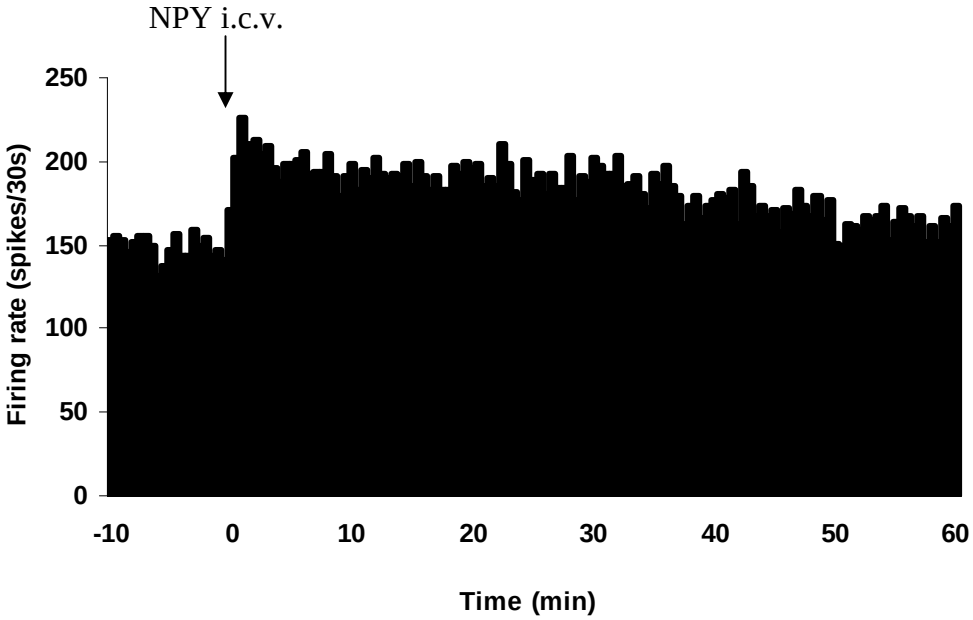

Fig. 3.3. Effect of central administration of NPY (5µg/2µl aCSF; i.c.v) on the firing rate of a SON oxytocin neurone in a virgin rat (Cell No. 93-1). Basal firing rate of 5 ± 0.05 spikes/s was increased by 2.6 spikes/s 1min after i.c.v. NPY.

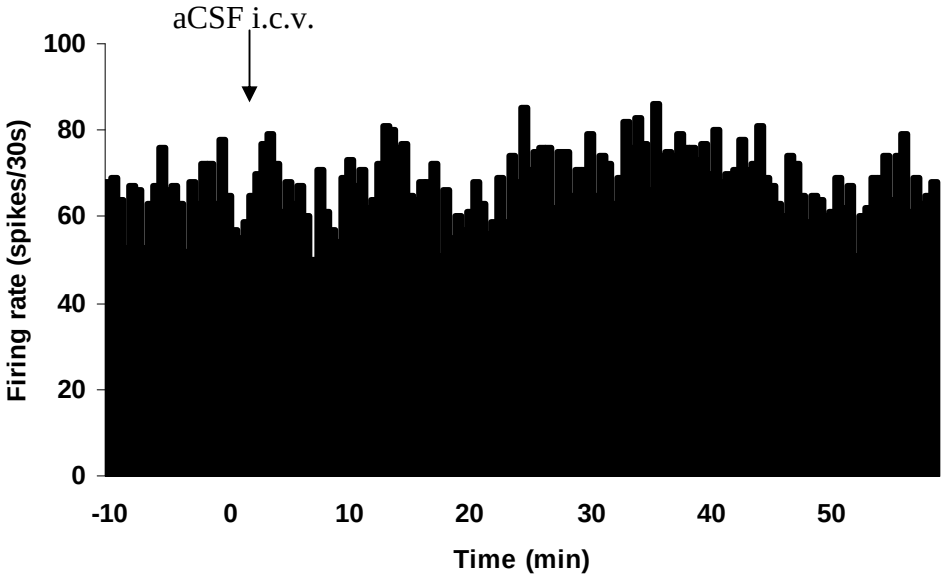

Fig. 3.4. Effect of central administration of vehicle (2µl aCSF; i.c.v) on the firing rate of a SON oxytocin neurone in a virgin rat (Cell No. 84-3). Basal firing rate: 2.2 ± 0.05 spikes/s.

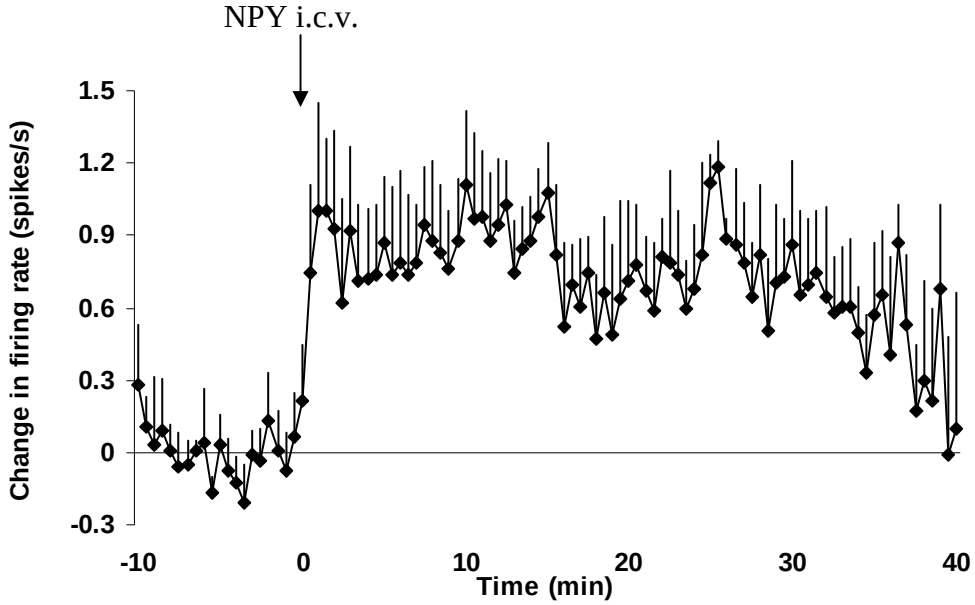

Fig. 3.5. Effect of central administration of NPY (5µg/2µl aCSF; i.c.v) on the firing rate of SON oxytocin neurones in virgin rats (n=5 cells; 4 rats). Values are mean ± s.e.m. Pre- *vs.* 0-30min post-NPY: P=0.01, paired t-test.

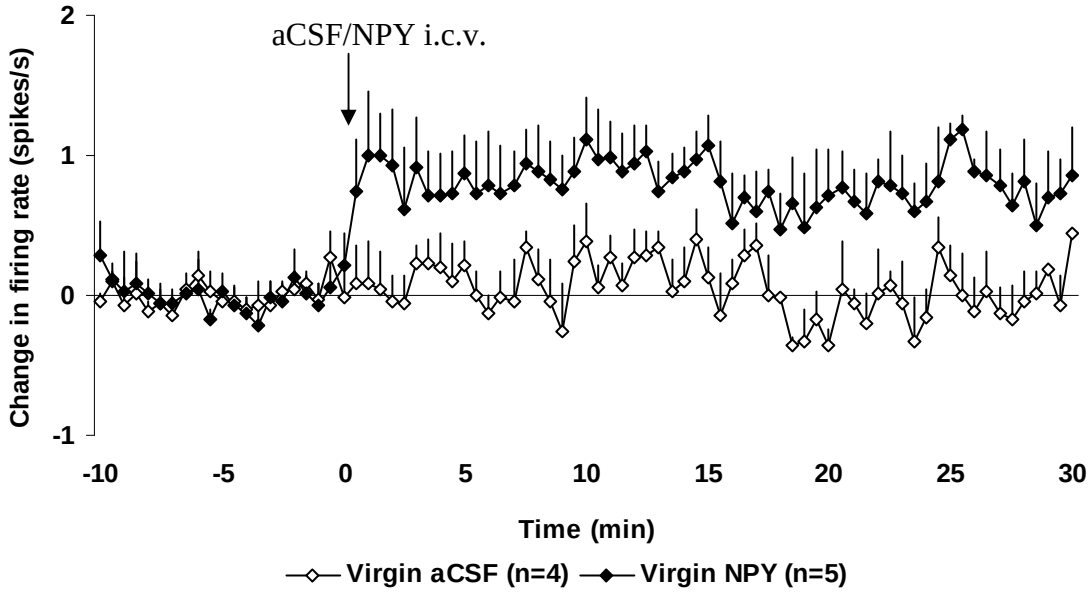

Fig. 3.6. Effect of central administration of NPY (5µg/2µl aCSF; i.c.v; n=5 cells; 4 rats) or vehicle (2µl aCSF; i.c.v; n=4 rats) on the firing rate of SON oxytocin neurones in virgin rats. Values are mean ± s.e.m. Change in firing rate during 0-30min after respective injections between virgin aCSF and virgin NPY groups: P=0.02, t-test. Virgin NPY group: P=0.01, paired t-test, pre- *vs.* 0-30min post-NPY within group. Virgin aCSF group: P=0.7, paired t-test, pre- *vs.* 0-10min after aCSF within group. Central administration of NPY significantly excited SON oxytocin neurones in virgin rats compared to vehicle control.

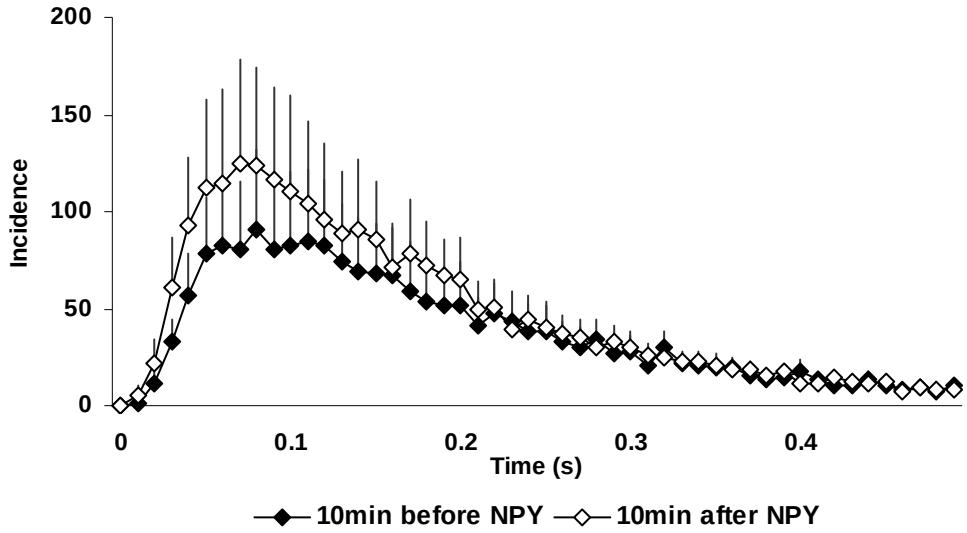

Fig. 3.7a. Effect of central administration of NPY (5µg/2µl aCSF; i.c.v) on the interspike interval histogram of SON oxytocin neurones in virgin rats (n=5 cells; 4 rats). Values are mean ± s.e.m; not normalised. Pre- *vs.* 10min post-NPY: P=0.049, paired t-test.

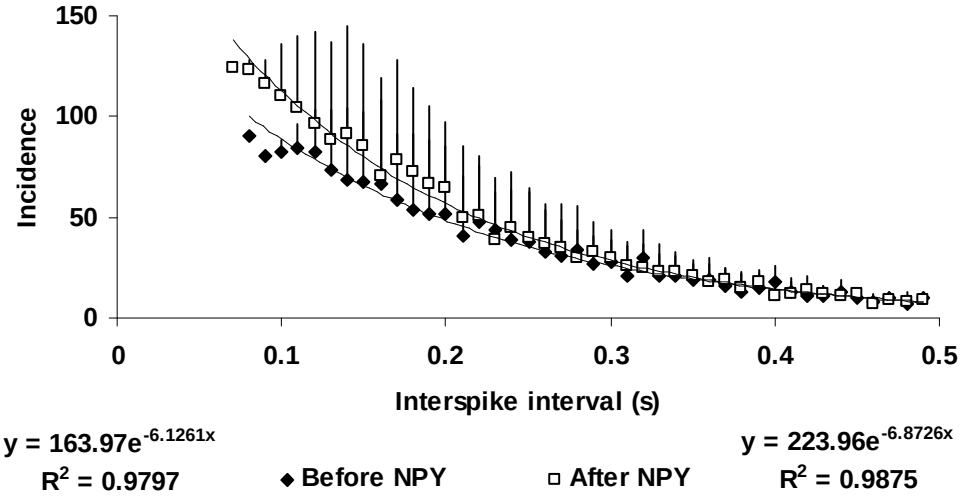

Fig. 3.7b. Interspike interval histogram fitted to exponential curves. Values are mean ± s.e.m. The regression equations are: before NPY: $y = 163.97e^{-6.1261x}$; after NPY: $y = 223.96e^{-6.8726x}$. R^2 values of 0.98 and 0.99 indicate that the exponential curves perfectly fit the data which is a characteristic of oxytocin neurones.

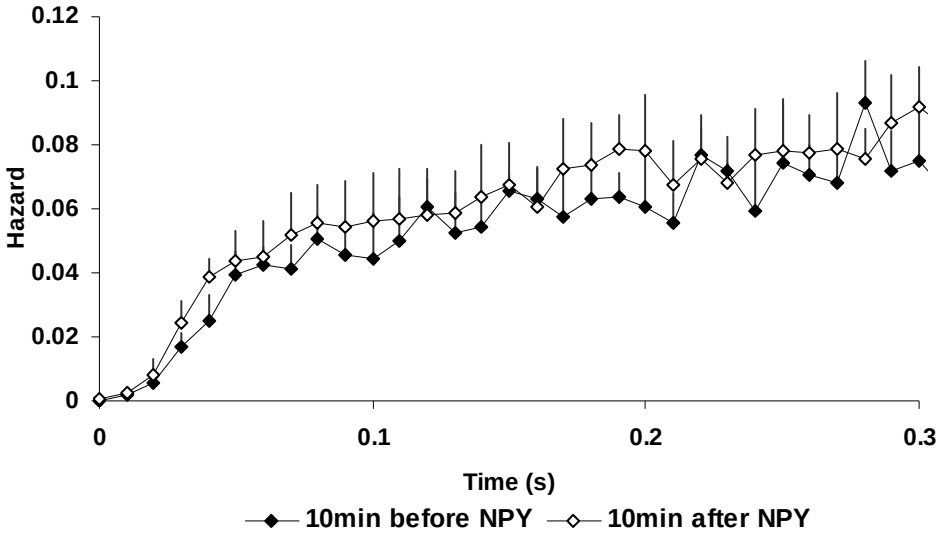

Fig. 3.8. Effect of central administration of NPY (5µg/2µl aCSF; i.c.v) on the mean post-spike probability of SON oxytocin neurones in virgin rats (n=5 cells; 4 rats): Hazard analysis. Values are mean ± s.e.m; not normalised.

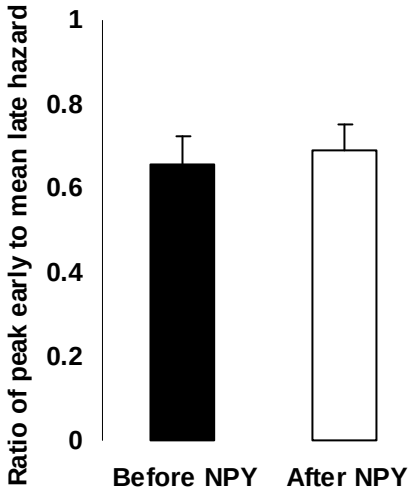

Fig. 3.9. Effect of central administration of NPY (5µg/2µl aCSF; i.c.v) on the mean post-spike probability of SON oxytocin neurones in virgin rats (n=5 cells; 4 rats): Ratio of peak early (<0.07s) to mean late (0.2 – 0.3s) hazard. Values are mean ± s.e.m. Before *vs.* after NPY: P=0.5, paired t-test. [n.s]

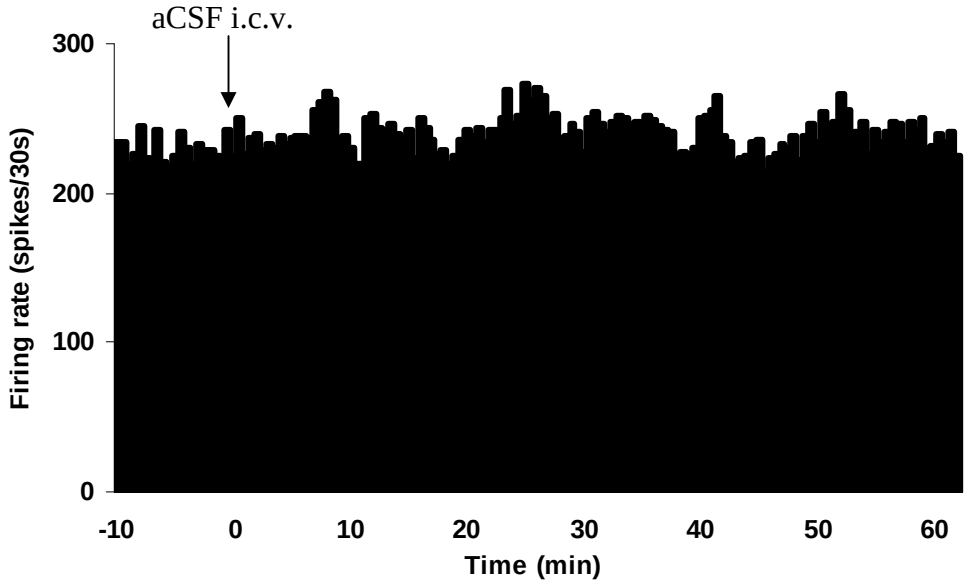

Fig. 3.10. Effect of central administration of vehicle (2µl aCSF; i.c.v) on the firing rate of a SON oxytocin neurone in a late pregnant rat (Cell No. 124P-3). Basal firing rate: 7.6 ± 0.07 spikes/s.

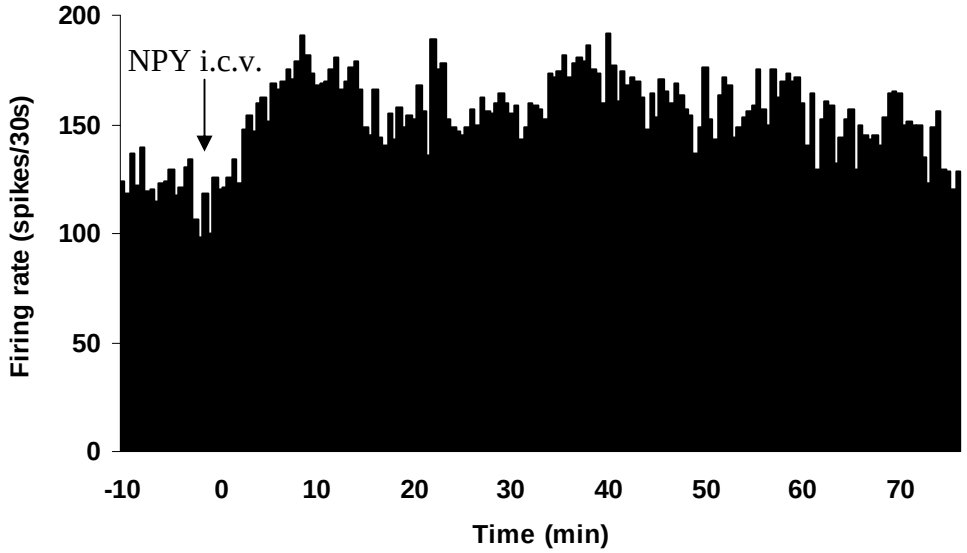

Fig. 3.11. Effect of central administration of NPY (5µg/2µl aCSF; i.c.v) on the firing rate of a SON oxytocin neurone in a pregnant rat (Cell No. 89P-3). Basal firing rate of 4 ± 0.08 spikes/s was increased by 2.3 spikes/s 8.5min after NPY.

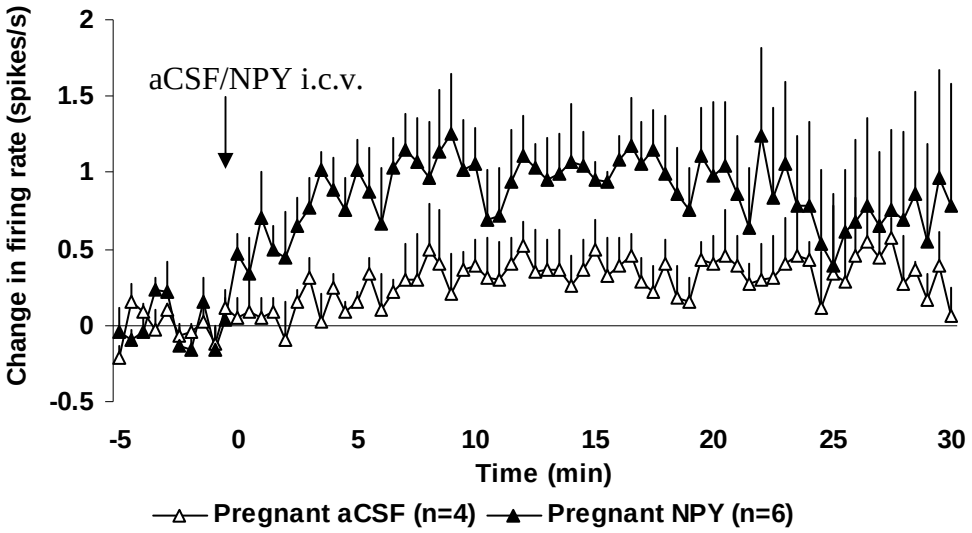

Fig. 3.12. Effect of central administration of NPY (5µg/2µl aCSF; i.c.v; n=6 rats) or vehicle (2µl aCSF; i.c.v; n=4 rats) on electrical activity of SON oxytocin neurones in late pregnant rats. Values are mean ± s.e.m. Pre- *vs.* 0-30min post-NPY within pregnant NPY group P=0.03, paired t-test. Pre- *vs.* 0-30min post-aCSF within pregnant aCSF group: P=0.053, paired t-test. P=0.048, t-test, 0-30min after aCSF/NPY between pregnant aCSF and pregnant NPY groups.

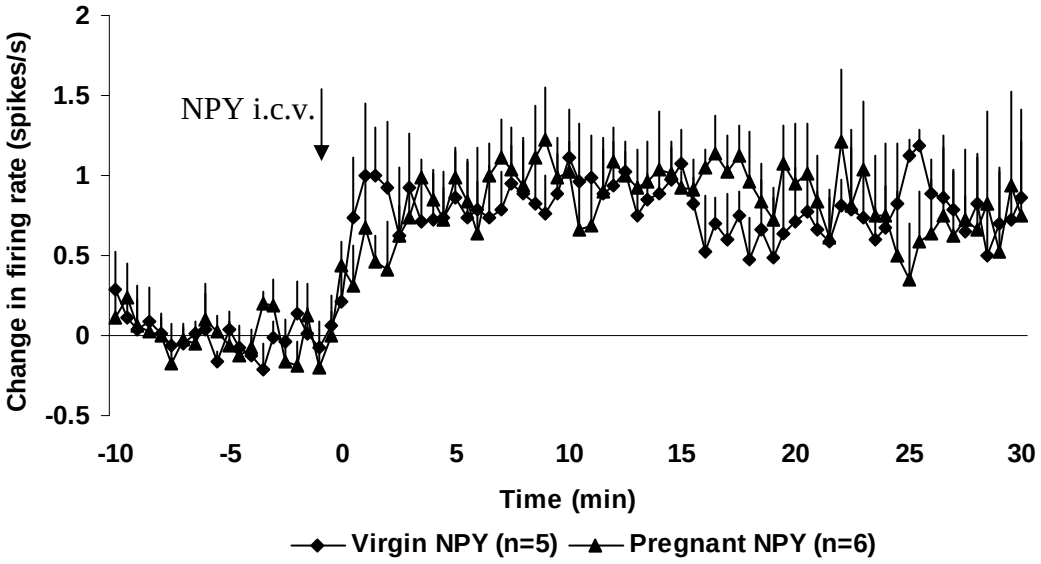

Fig. 3.13. Effect of central administration of NPY (5µg/2µl aCSF) on electrical activity of SON oxytocin neurones in virgin (n=5 cells; 4 rats) and late pregnant (n=6 rats) rats. Values are mean ± s.e.m. Pre- *vs.* 0-30min after NPY within virgin NPY group: P=0.01, paired t-test. Pre- *vs.* 0-30min after NPY within pregnant NPY group: P=0.03, paired t-test. 0-30min after NPY between virgin NPY and pregnant NPY groups: P=0.9, t-test.

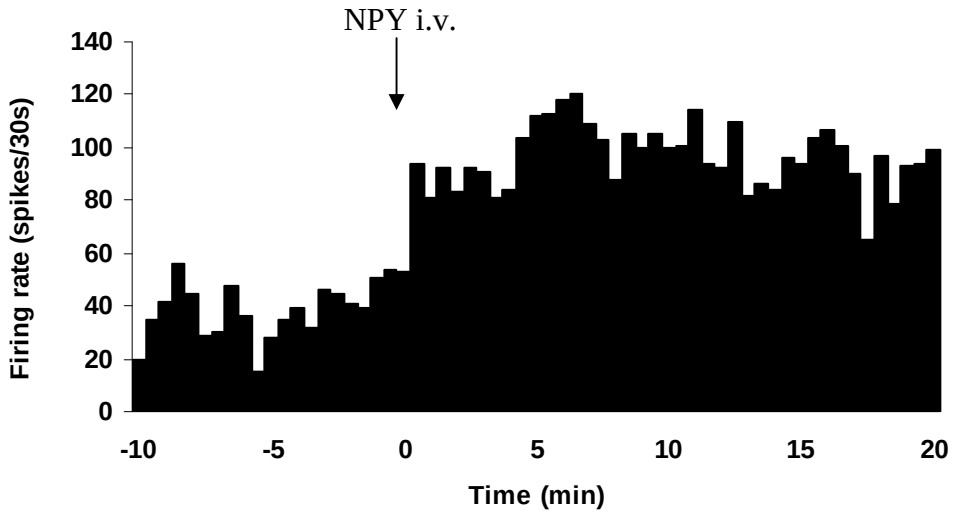

Fig. 3.14. Effect of systemic administration of NPY (20µg; i.v) on the firing rate of a SON oxytocin neurone in a virgin rat (Cell No. 25-10). Basal firing rate of 1.3 spikes/s was increased by 2.7 spikes/s 6min after NPY.

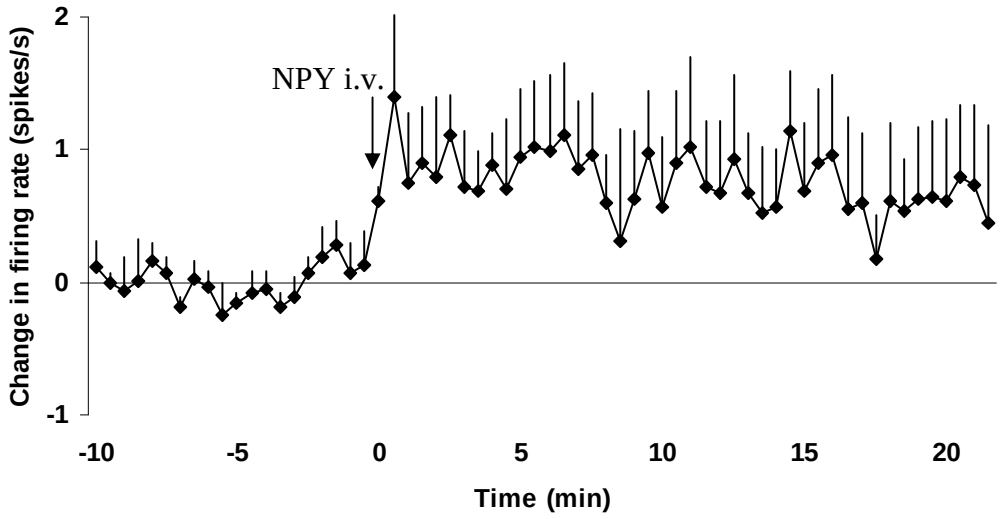

Fig. 3.15. Effect of systemic administration of NPY (20µg/rat; i.v) on the firing rate of SON oxytocin neurones in virgin rats (n=5 rats). Values are mean ± s.e.m. P=0.04, paired t-test, pre- vs 0-5min post- NPY.

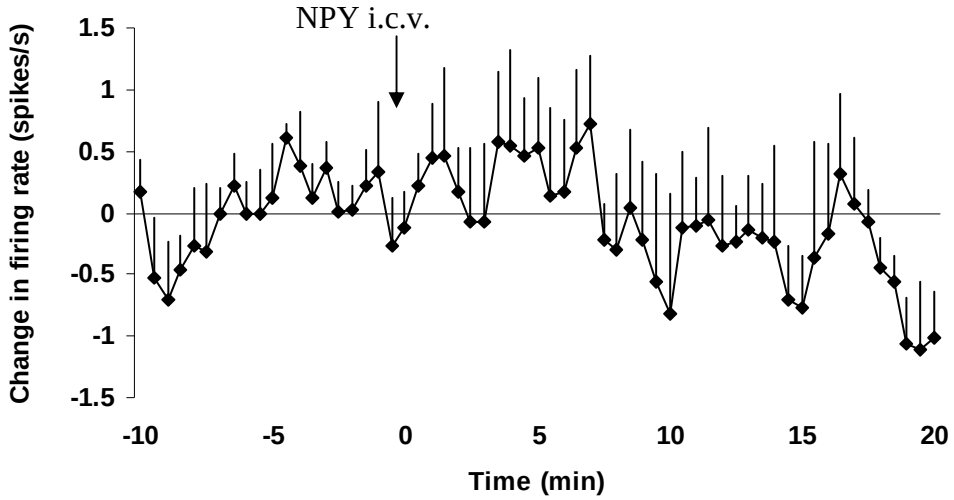

Fig. 3.16. Effect of central administration of NPY (5µg/2µl; i.c.v) on firing rate of SON vasopressin neurones in virgin rats (n=4 rats). Values are mean ± s.e.m. Pre- vs 0-10min post-NPY: P=0.6, paired t-test.

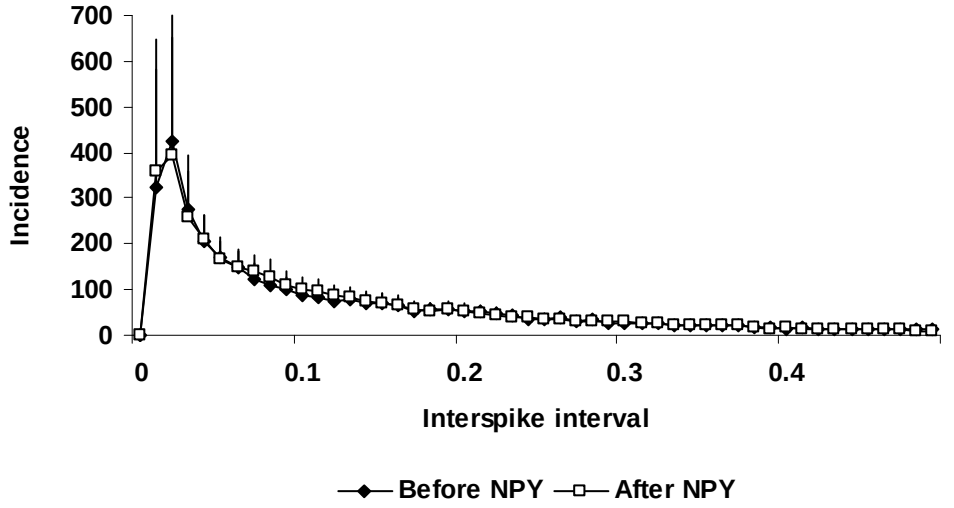

Fig. 3.17a. Effect of central administration of NPY (5µg/2µl; i.c.v) on the interspike interval histogram of SON vasopressin neurones in virgin rats (n=5 rats). Values are mean ± s.e.m; not normalised. There was no change in the firing intervals after NPY.

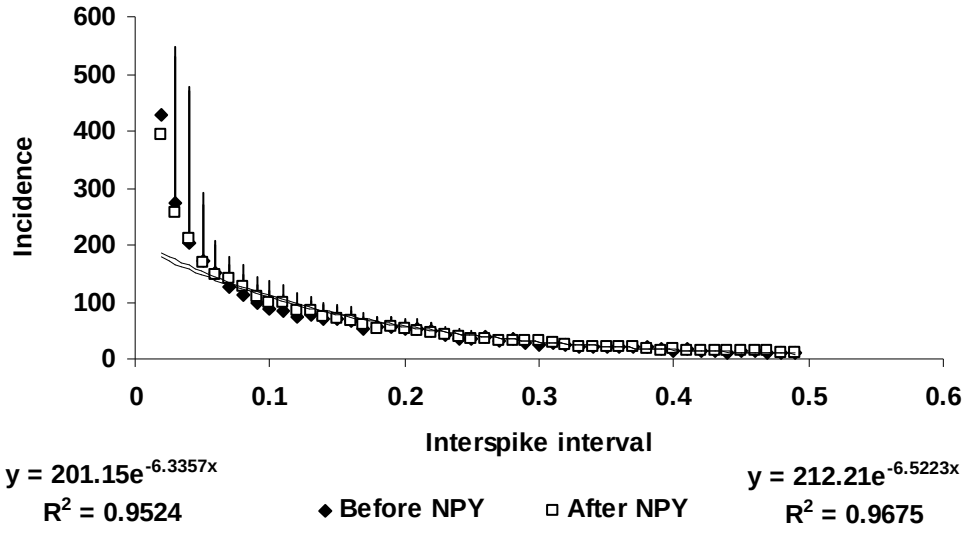

Fig. 3.17b. Interspike interval histograms of vasopressin neurones before and after central NPY administration fitted to exponential curves. Values are mean ± s.e.m. The distal tail of the histograms fit well to exponential lines, indicated by the R^2 values of 0.95 and 0.97, but the intervals less than 0.2s did not fit, which is a characteristic of vasopressin neurones.

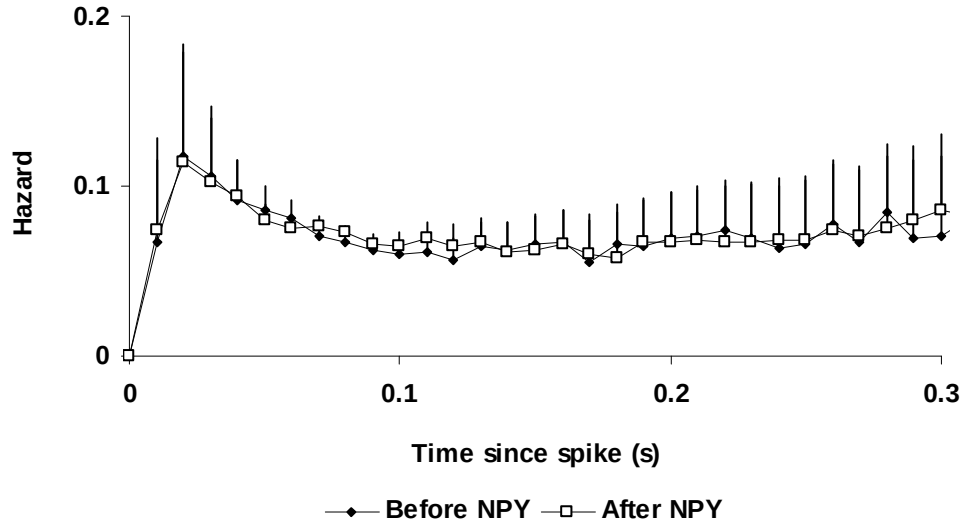

Fig. 3.18. Effect of central administration of NPY (5µg/2µl; i.c.v) on the mean post-spike probability of SON vasopressin neurones in virgin rats (n=5 rats): Hazard analysis. Values are mean ± s.e.m; not normalised. There was no change in the shape of the hazard plot after NPY.

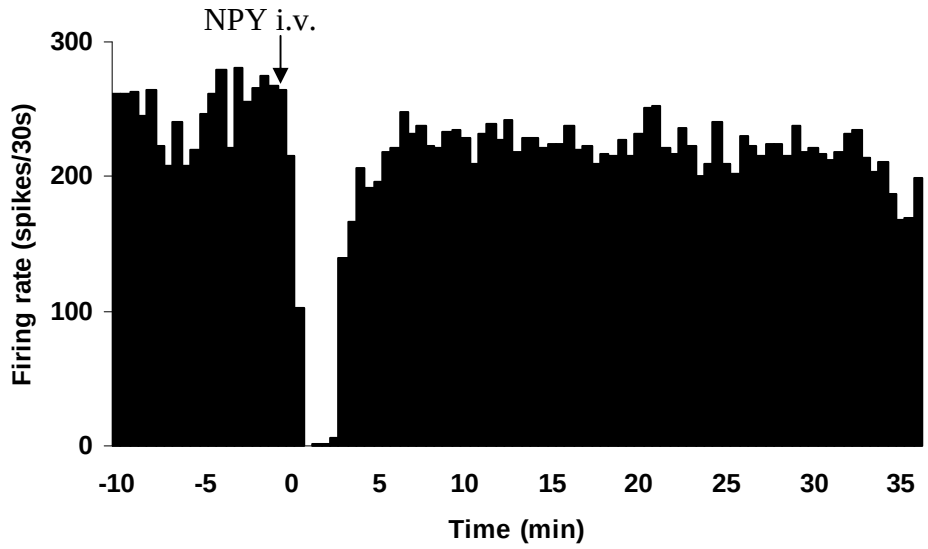

Fig. 3.19. Effect of systemic administration of NPY (20µg/rat; i.v) on the firing rate of a non-phasic SON vasopressin neurone in a virgin rat (Cell No. 15-1). Basal firing rate was 8.4 spikes/s. The activity was inhibited by systemic NPY administration for ca. 2 min.

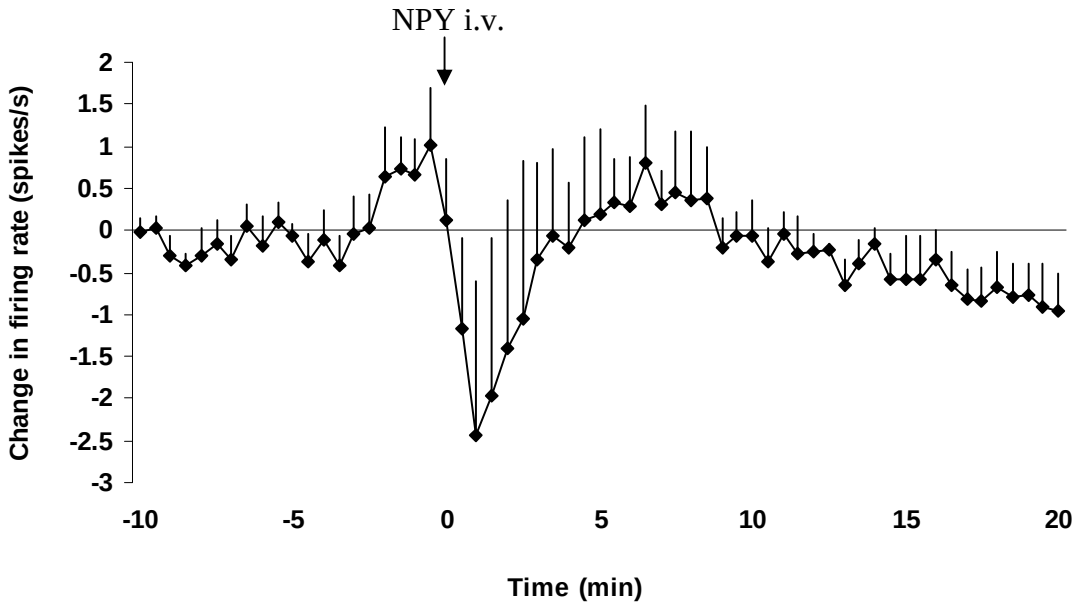

Fig. 3.20. Effect of systemic administration of NPY (20µg/rat; i.v) on the firing rate of non-phasic SON vasopressin neurones in virgin rats (n=6 rats). Values are mean ± s.e.m. Pre- *vs.* 0-5min post-NPY: P=0.48, paired t-test. [n.s]

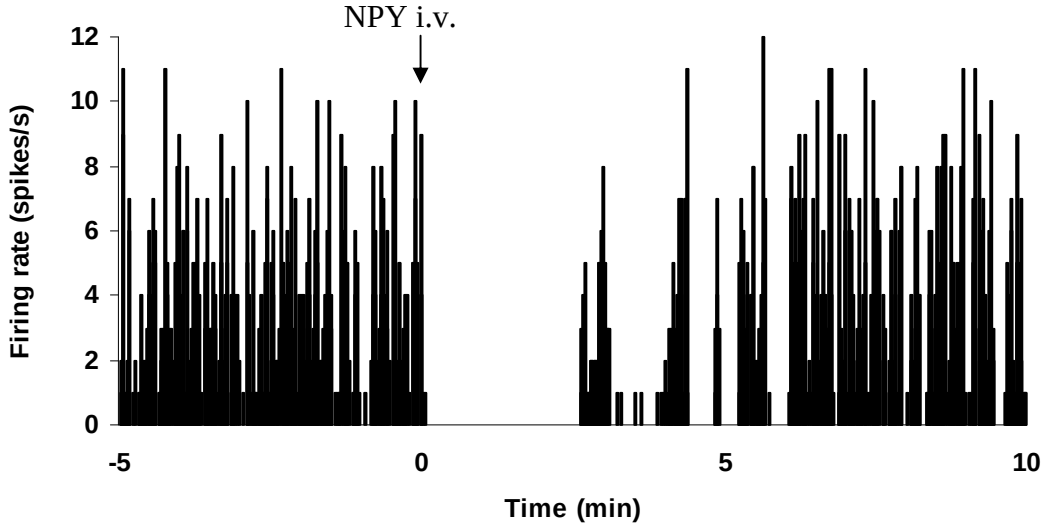

Fig. 3.21. Effect of systemic administration of NPY (20µg/rat; i.v) on the firing rate of a phasic SON vasopressin neurone in a virgin rat (Cell No. 9-5). Activity quotient and frequency within bursts decreased from 0.2 (basal) to 0.1 and 3.2 spikes/s (basal) to 2.9 spikes/s, respectively, 0-5min after NPY, while the mean interburst interval increased from 7.2s (basal), to 51.9s 0-5min after NPY administration.

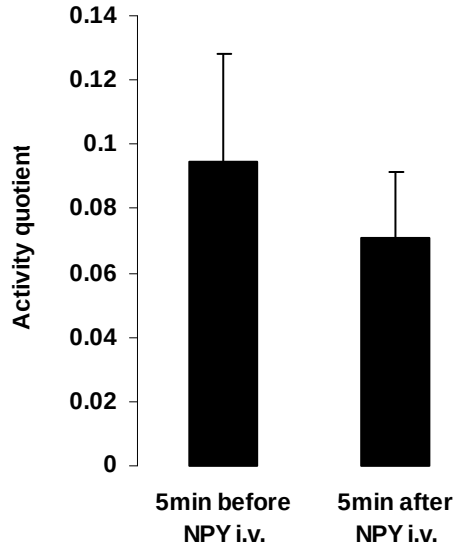

Fig. 3.22. Effect of systemic administration of NPY (20µg/rat; i.v) on the firing rate of phasic SON vasopressin neurones in virgin rats (n=3 rats): Activity quotient. Values are mean ± s.e.m. Pre- vs post-NPY: P=0.3, paired t-test. [n.s]

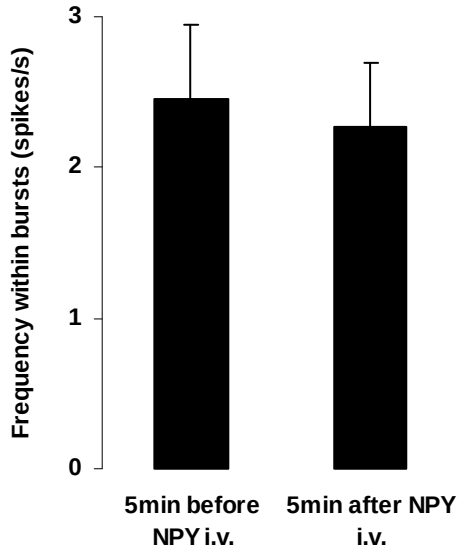

Fig. 3.23. Effect of systemic administration of NPY (20µg/rat; i.v) on the firing rate of phasic SON vasopressin neurones in virgin rats (n=3 rats): Frequency within bursts. Values are mean ± s.e.m. Pre- vs post-NPY: P=0.08, paired t-test. [n.s]

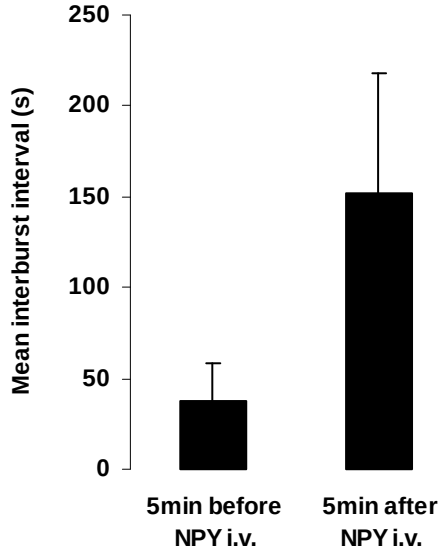

Fig. 3.24. Effect of systemic administration of NPY (20µg/rat; i.v) on the firing rate of phasic SON vasopressin neurones in virgin rats (n=3 rats): Mean interburst interval. Values are mean ± s.e.m. Pre- vs post-NPY: P=0.12; paired t-test. [n.s]

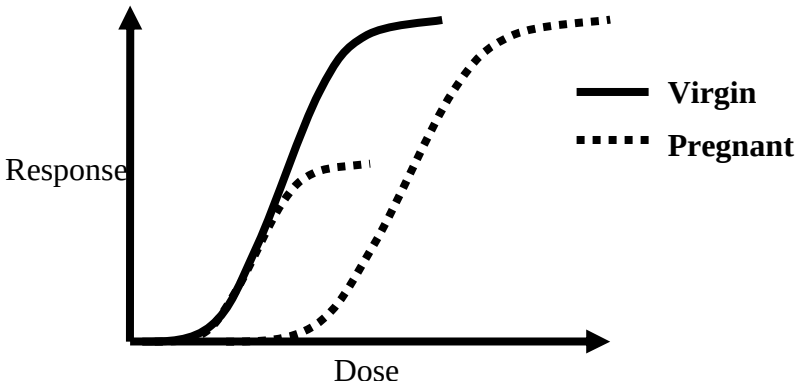

Fig. 4.1. Hypothesis for response to leptin in pregnant rats. As oxytocin neurone responses to various stimuli are attenuated during pregnancy in rats, it was hypothesized that leptin-induced effects on SON oxytocin neurones are also suppressed in pregnant rats. Hence, a reduction in the response to a particular dose of leptin or shift to right in the dose response curve is expected for pregnant rats.

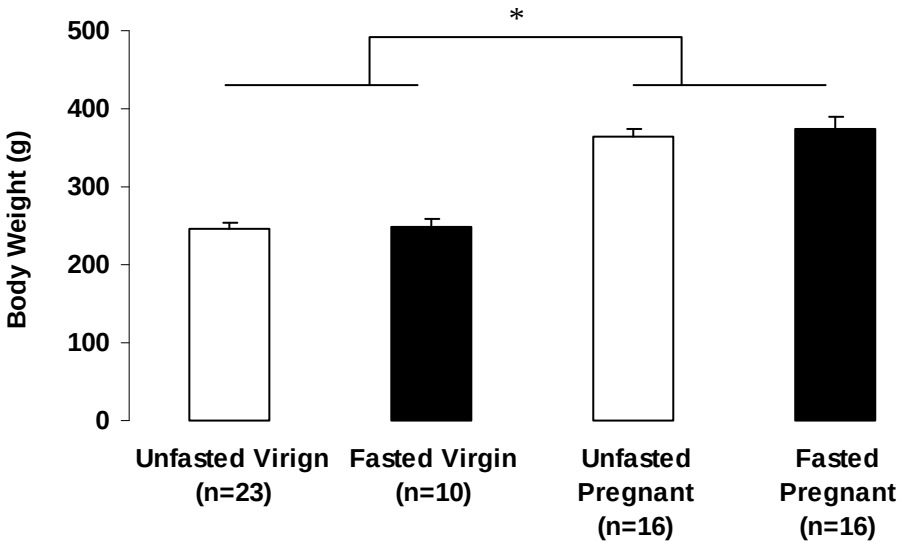

Fig. 4.2. The mean body weights of fasted/unfasted virgin and pregnant rats used in leptin study. Values are mean ± s.e.m. *P<0.001, $F_{1, 62}$=124.9, two-way ANOVA, virgin *vs.* pregnant groups. Fasting for 18h did not alter the body weight significantly within virgin (fasted virgin *vs.* unfasted virgin: P=0.7, Mann-Whitney rank sum test) or pregnant (fasted pregnant *vs.* unfasted pregnant: P=0.5, t-test) groups.

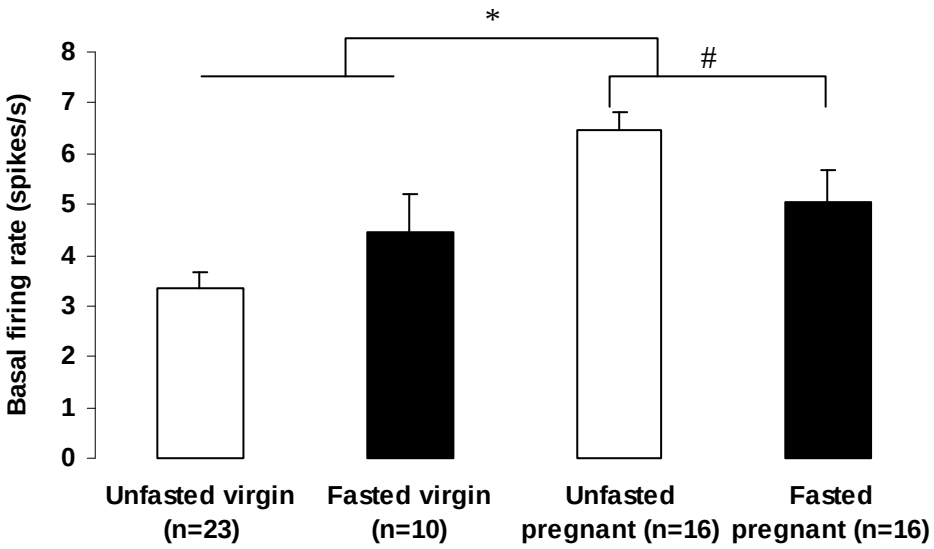

Fig. 4.3. Basal firing rates of SON oxytocin neurones in the four groups of rats used in leptin study. Values are mean ± s.e.m. *P<0.001, $F_{1, 61}$=15.2, two-way ANOVA, virgin *vs.* pregnant groups. #P=0.035, Two-way ANOVA followed by all pair-wise multiple comparison procedure (Holm-Sidak method), unfasted pregnant *vs.* fasted pregnant groups. There is a significant interaction between feeding (unfasted/fasted) and reproductive (virgin/pregnant) status (P=0.01, $F_{1, 61}$=6.9, two-way ANOVA).

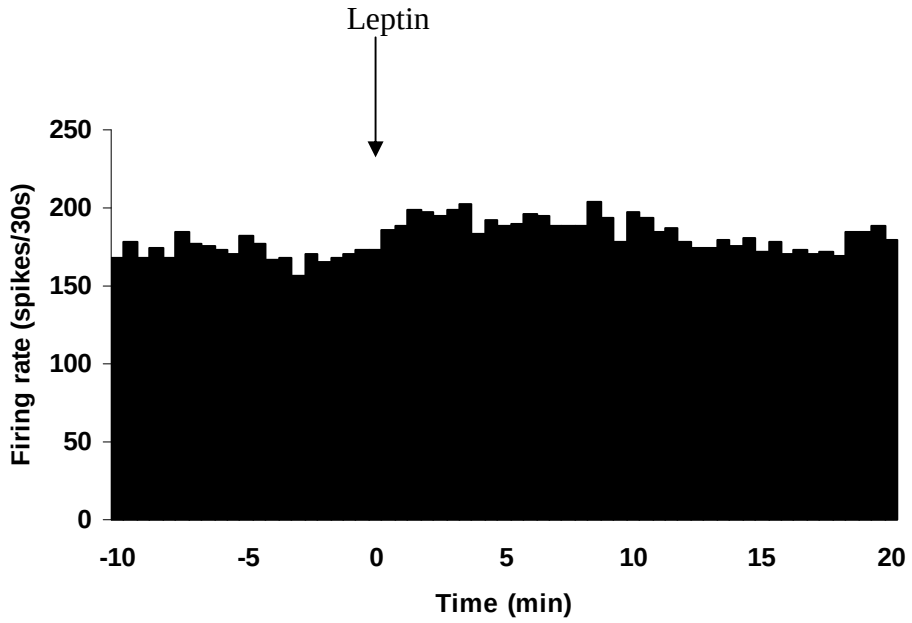

Fig. 4.4. Effect of systemic administration of leptin (100µg; i.v) on electrical activity of a SON oxytocin neurone in an unfasted virgin rat (Cell No. 188-3). The basal firing rate of 5.7 ± 0.05 spikes/s was increased by 1 spike/s 3.5min after leptin and the rate returned to basal by 12min.

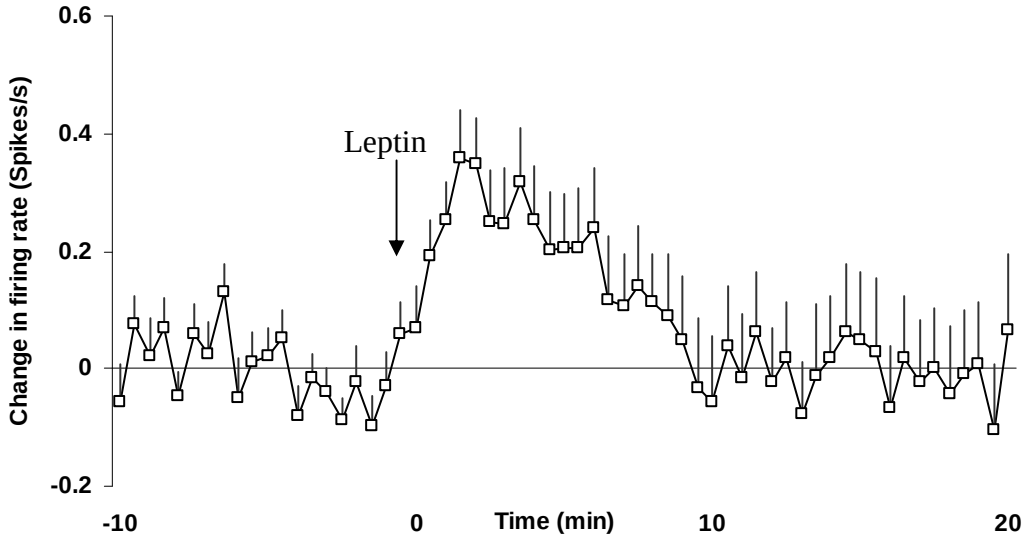

Fig. 4.5. Effect of systemic administration of leptin (100µg; i.v) on electrical activity of SON oxytocin neurones in unfasted virgin rats (n=23). Values are means ± s.e.m. of average changes from across 10min of basal in 30s bins. Pre- *vs.* 10min post-leptin: P=0.01, paired t-test.

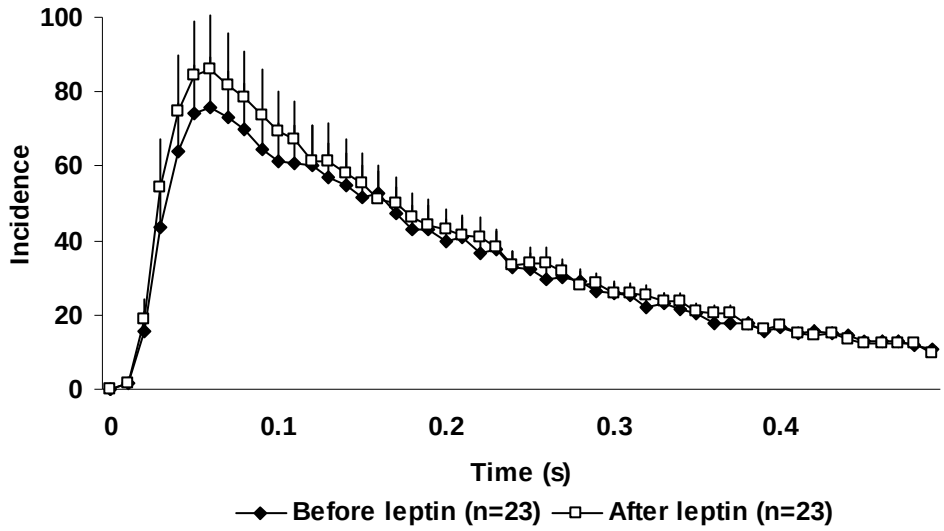

Fig. 4.6a. Effect of systemic administration of leptin (100µg; i.v) on mean interspike interval of SON oxytocin neurones 10min before and 10min after administration of leptin in unfasted virgin rats (n=23). Values are mean ± s.e.m; not normalised. Pre- *vs*. 0-10min post-leptin P=0.004, paired t-test.

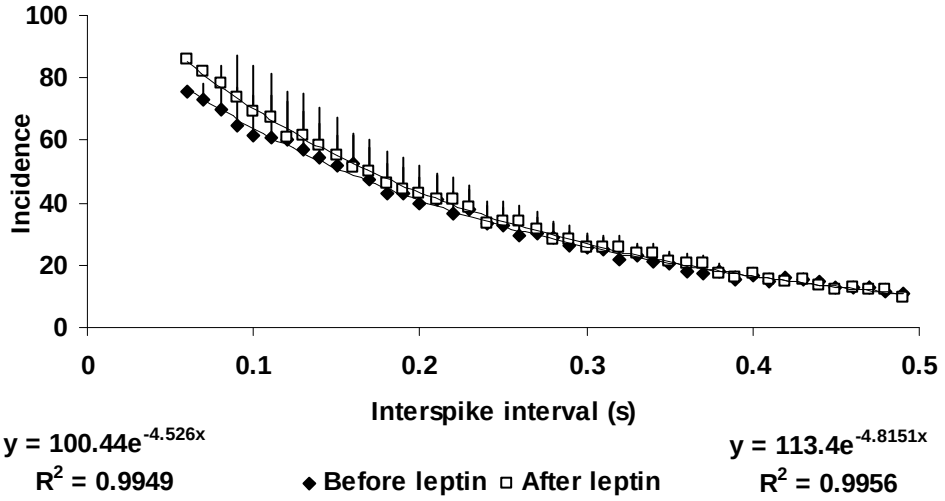

Fig. 4.6b. The interspike interval histograms before and after leptin fitted to the exponential curves. R^2 value of 0.99 indicates that the regression lines perfectly fit the data.

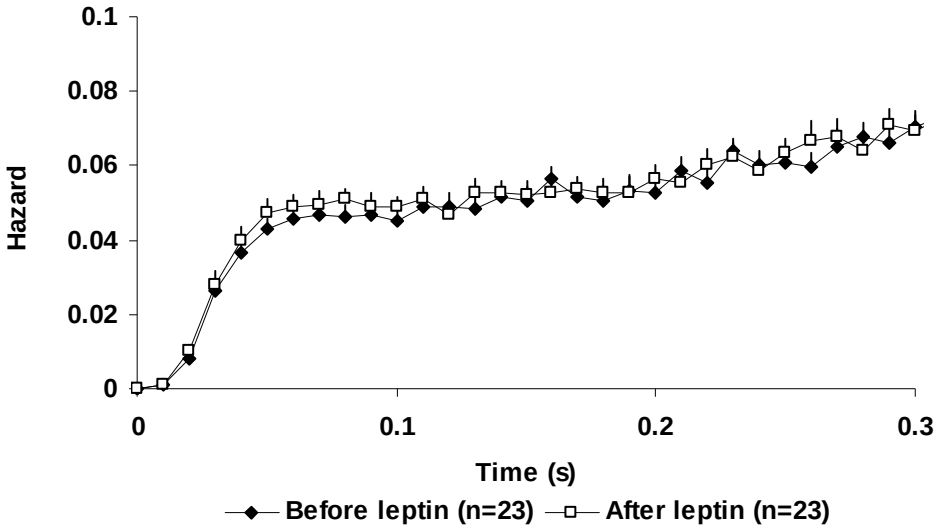

Fig. 4.7. Effect of systemic administration of leptin (100µg; i.v) on mean post-spike probability of SON oxytocin neurones 10min before and 10min after administration of leptin in unfasted virgin rats (n=23): Hazard analysis. Values are mean ± s.e.m; not normalised. There is no change in the shape of the hazard plot after administration of leptin.

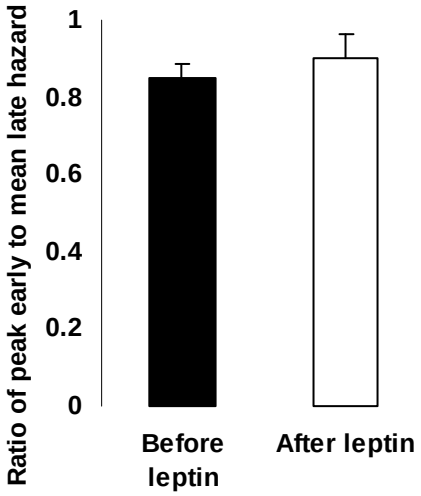

Fig. 4.8. Effect of systemic administration of leptin (100µg; i.v) on mean post-spike probability of SON oxytocin neurones in unfasted virgin rats (n=23): Ratio of peak early (<0.07s) to mean late (0.2-0.3s) hazard 10min before and 10min after administration of leptin. Values are mean ± s.e.m. Pre- vs post-leptin: P=0.6, Wilcoxon signed rank test. [n.s]

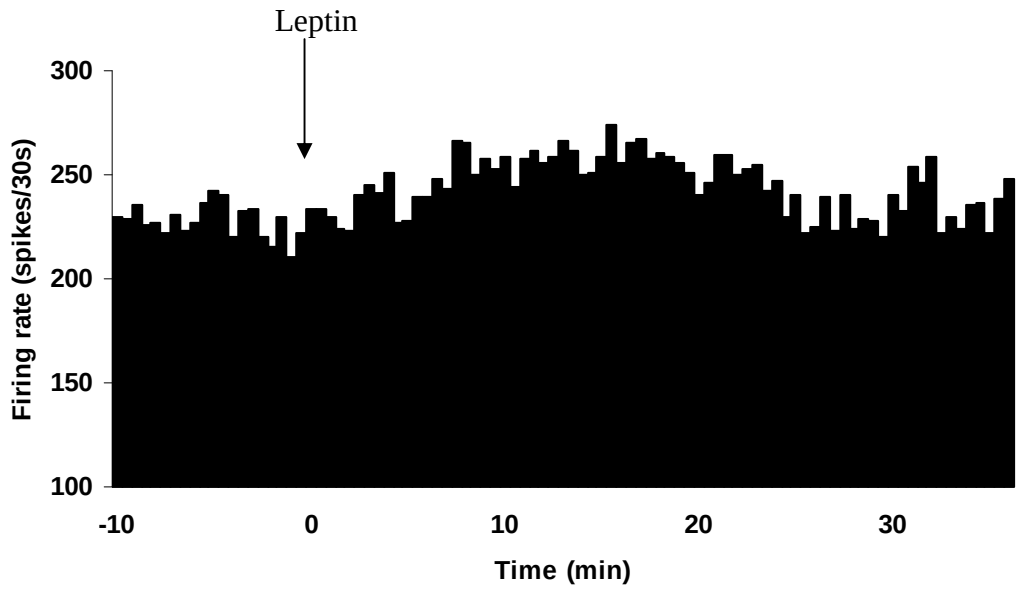

Fig. 4.9. Effect of systemic administration of leptin (100µg; i.v) on electrical activity of SON oxytocin neurones in an unfasted pregnant rat (No. 142P-2). The basal rate of 7.6 ± 0.06 spikes/s was increased by 1.5 spikes/s 15.5min after leptin treatment. The rate returned to basal by 25min.

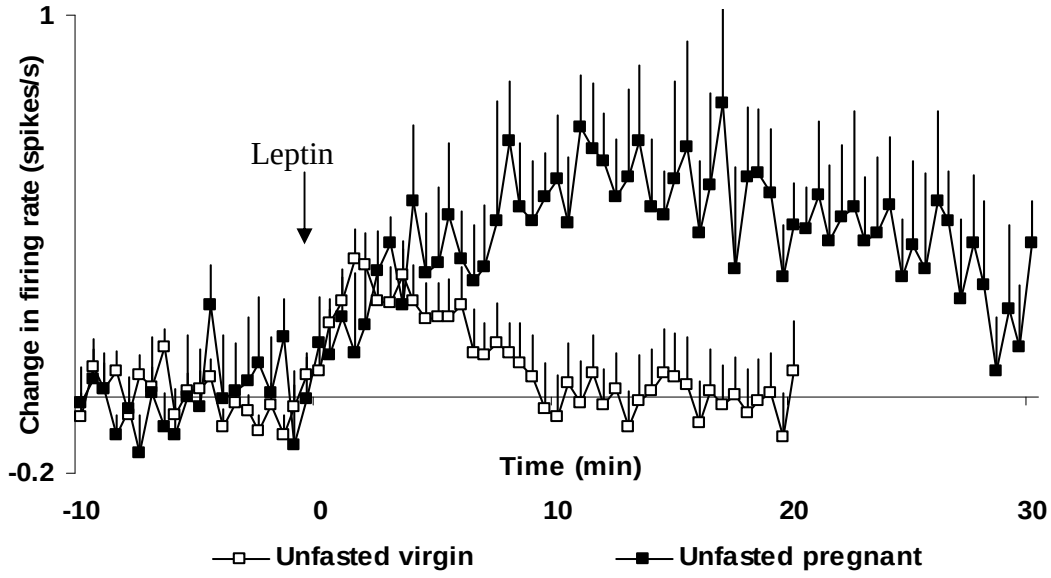

Fig. 4.10. Effect of systemic administration of leptin (100µg; i.v) on electrical activity of SON oxytocin neurones in unfasted virgin (n=23) and pregnant (n=5) rats. Values are means ± s.e.m. of average changes from across 10min of basal in 30s bins. The oxytocin neurones in the unfasted virgin groups were recorded only for 20min after leptin administration and hence the data appears truncated. Pre- vs 0-10min post-leptin within unfasted virgin group: P=0.01, paired t-test; pre- vs 0-20 or 0-30min post-leptin within unfasted pregnant group: P=0.006, paired t-test; 10-20min after leptin between unfasted virgin and pregnant groups: P=0.01, t-test.

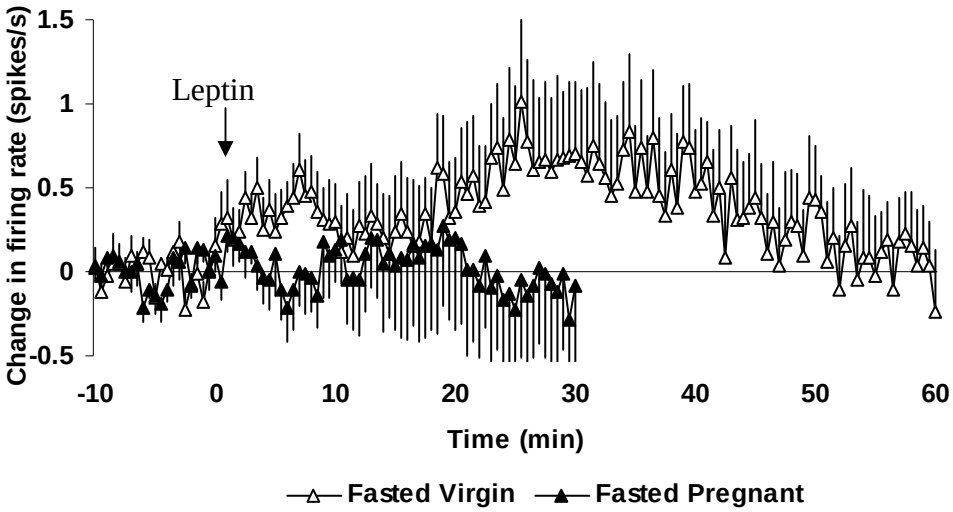

Fig. 4.11a. Effect of systemic administration of leptin (100µg; i.v) on electrical activity of SON oxytocin neurones in fasted virgin (n=8) and fasted pregnant (n=8) rats. Values are means ± s.e.m. of average changes from across 10 min of basal in 30 s bins. Some of the oxytocin neurones in the fasted pregnant rats were lost 30min after leptin and hence the data was truncated. Pre- *vs.* 0-10min post leptin within the fasted virgin group: P=0.051, paired t-test; pre *vs.* 20-30min after leptin within the fasted virgin group: P=0.2, paired t-test; pre- *vs.* 0-10min after leptin within the fasted pregnant group: P=0.8, paired t-test; fasted virgin *vs.* fasted pregnant: 20-30min after leptin between groups: P=0.18, t-test. [n.s]

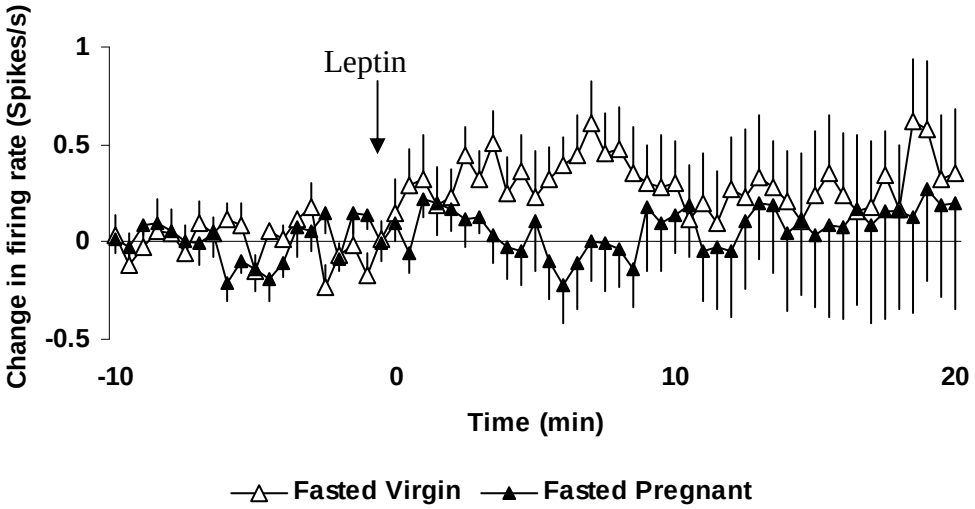

Fig. 4.11b. Effect of systemic administration of leptin (100µg; i.v) on electrical activity of SON oxytocin neurones in fasted virgin (n=8) and pregnant (n=8) rats during the first 0-20min period after leptin injection. Values are means ± s.e.m. of average changes from across 10 min of basal in 30 s bins. Pre- *vs.* 0-10min post leptin within fasted virgin group: P=0.051, paired t-test; fasted pregnant: pre- *vs.* 0-10min after leptin within the group: P=0.8, paired t-test; fasted virgin *vs.* fasted pregnant 0-10min after leptin between groups: P=0.25, t-test. [n.s]

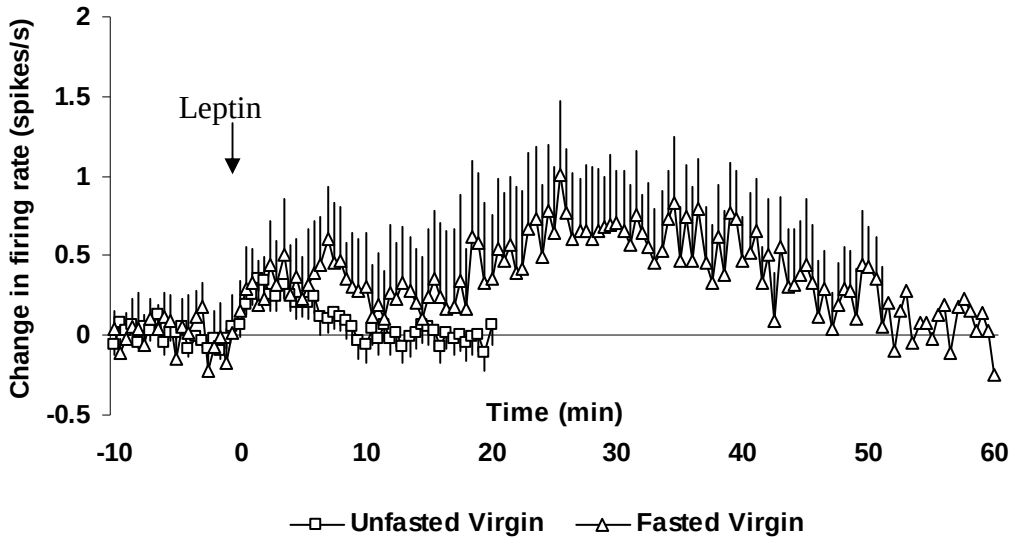

Fig. 4.12a. Effect of systemic administration of leptin (100µg; i.v) on electrical activity of SON oxytocin neurones in unfasted (n=23) and fasted virgin (n=8) rats. Values are means ± s.e.m of average changes from across 10min of basal in 30s bins. The oxytocin neurones in the unfasted virgin group were recorded only until 20min after leptin and hence the data appears truncated. Pre- *vs.* 0-10min post-leptin within unfasted virgin group: P=0.01, paired t-test. Pre- *vs.* 0-10min post-leptin in fasted virgin rats: P=0.051, paired t-test (pre- *vs.* 20-30min post-leptin: P=0.2, paired t-test). The response after leptin did not differ significantly between groups (10-20min after leptin: P=0.1, t-test).

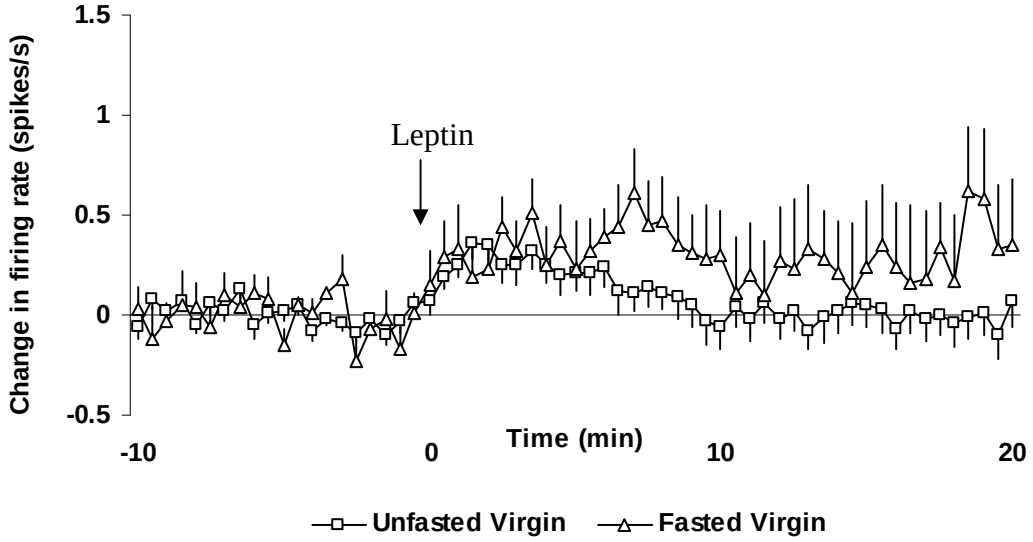

Fig. 4.12b. Effect of systemic administration of leptin (100µg; i.v) on electrical activity of SON oxytocin neurones in unfasted (n=23) and fasted (n=8) virgin rats during the initial 0-20min after leptin. Values are means ± s.e.m of average changes from across 10min of basal in 30s bins. Pre- *vs.* 0-10min post-leptin within unfasted virgin group: P=0.01, paired t-test. Pre- *vs.* 0-10min post-leptin in fasted virgin rats: P=0.051, paired t-test. The response after leptin did not differ significantly between groups (10-20min after leptin: P=0.1, t-test).

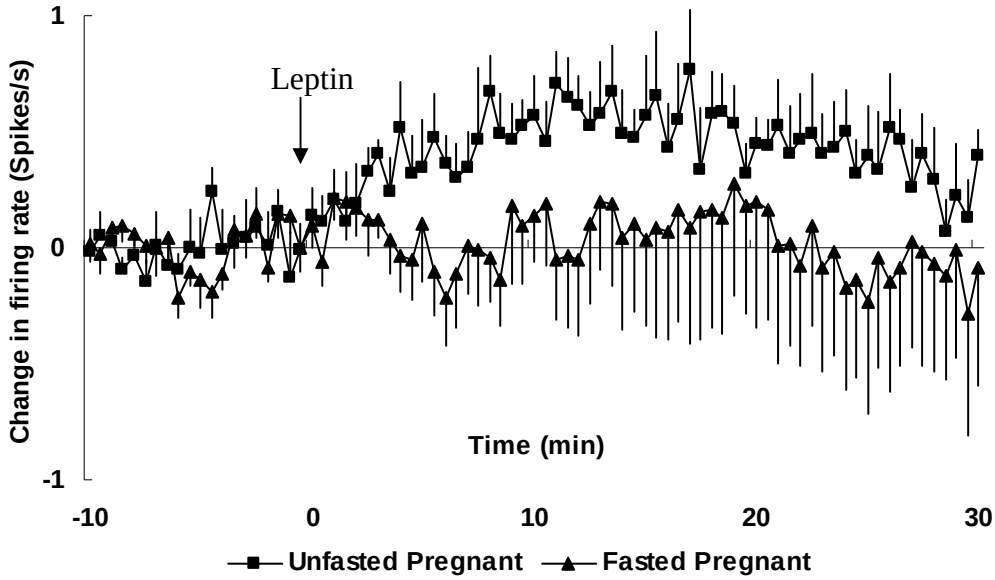

Fig. 4.13. Effect of systemic administration of leptin (100µg; i.v) on electrical activity of SON oxytocin neurones in unfasted (n=5) and fasted (n=8) pregnant rats. Values are means ± s.e.m. of average changes from across 10min of basal in 30s bins. Pre- *vs.* 0-20min (and 0-30min) post-leptin within unfasted pregnant group: P=0.006, paired t-test. Pre- *vs.* 0-10min post-leptin within fasted pregnant group: P=0.8, paired t-test. 0-10min and 0-20min after leptin between fasted and unfasted pregnant groups: P=0.1 and P=0.3, t-test.

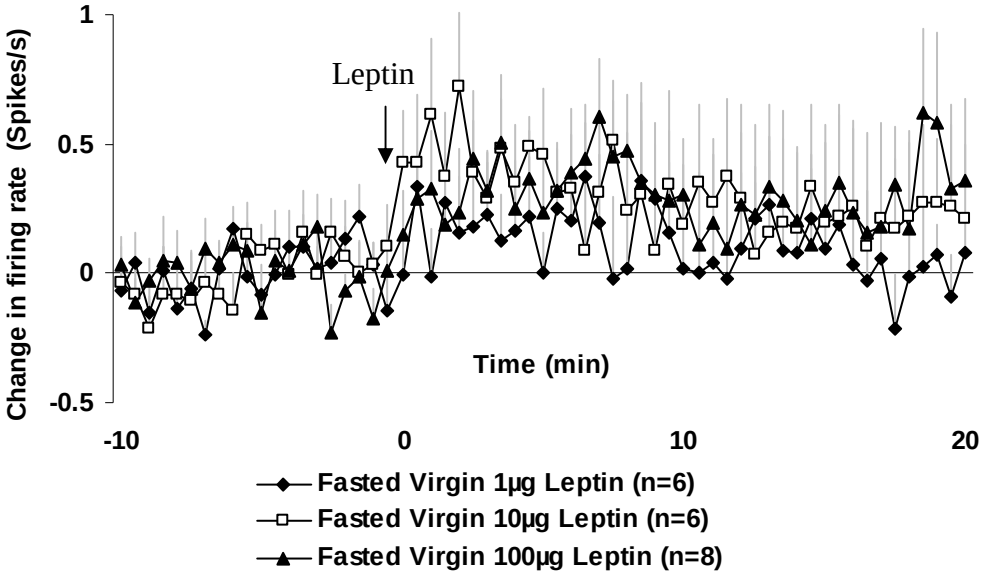

Fig. 4.14. Effect of systemic administration of different doses of leptin on electrical activity of SON oxytocin neurones in fasted virgin rats. Values are means ± s.e.m. of average changes from across 10min of basal in 30s bins. The basal firing rates were 4.2 ± 0.8 spikes/s, 4.55 ± 0.8 spikes/s and 4.45 ± 0.75 spikes/s, respectively, in the 1, 10 and 100µg leptin groups and they did not differ (P=0.98, one-way ANOVA). Pre- *vs.* 0-10min post-leptin within 100µg leptin group P=0.051, paired t-test. Pre- *vs.* 0-10min post-leptin: 1µg: P=0.2, 10µg: P=0.08, paired t-test. 0-10min after leptin between groups: P=0.6, one-way ANOVA.

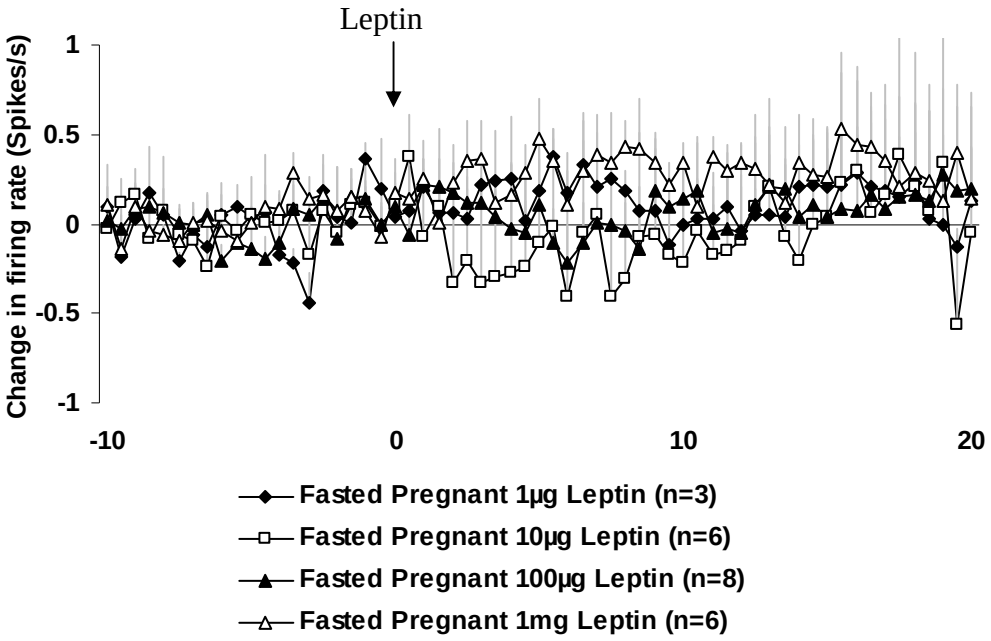

Fig. 4.15. Effect of systemic administration of different doses of leptin on electrical activity of SON oxytocin neurones in fasted pregnant rats. Values are means ± s.e.m. of average changes from across 10 min of basal in 30 s bins. Basal firing rates: 4.3 ± 2.1 spikes/s (1µg), 6 ± 1.1 spikes/s (10µg), 4.6 ± 0.7 spikes/s (100µg) and 5.9 ± 1 spikes/s (1mg); they did not differ (P=0.6, one-way ANOVA). Pre- *vs*. 0-10min post-leptin within groups: 1µg: P=0.3; 10µg: P=0.7; 100µg: P=0.8; 1mg: P=0.1, paired t-test. 0-10min post-leptin between groups: P=0.8, one-way ANOVA. [n.s]

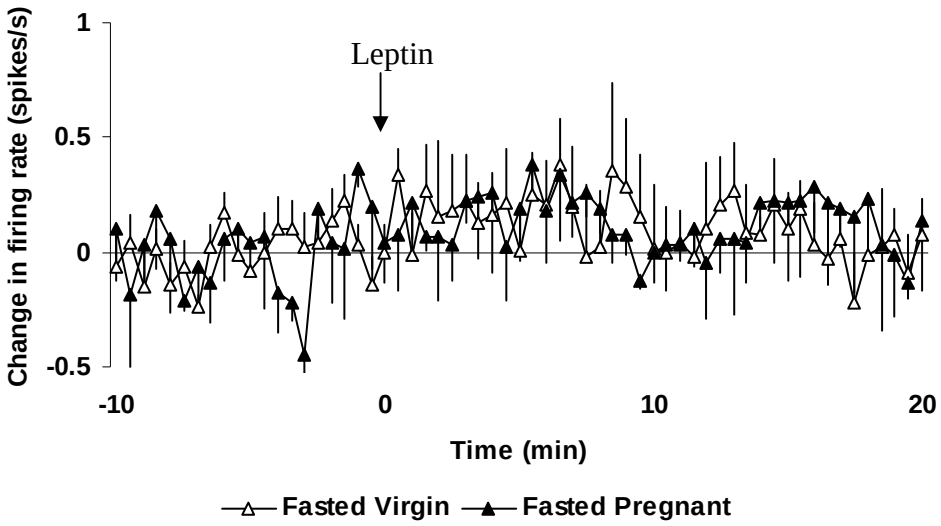

Fig. 4.16a. Effect of systemic administration of leptin (1µg; i.v) on electrical activity of SON oxytocin neurones in fasted virgin (n=6) and fasted pregnant (n=3) rats. Values are means ± s.e.m. of average changes from across 10 min of basal in 30 s bins. Pre- *vs.* 0-10min post-leptin within fasted virgin group: P=0.2, paired t-test. Pre- *vs.* 0-10min post-leptin within fasted pregnant group: P=0.3, paired t-test. 0-10min after leptin between fasted virgin and pregnant groups: P=0.9, t-test. [n.s]

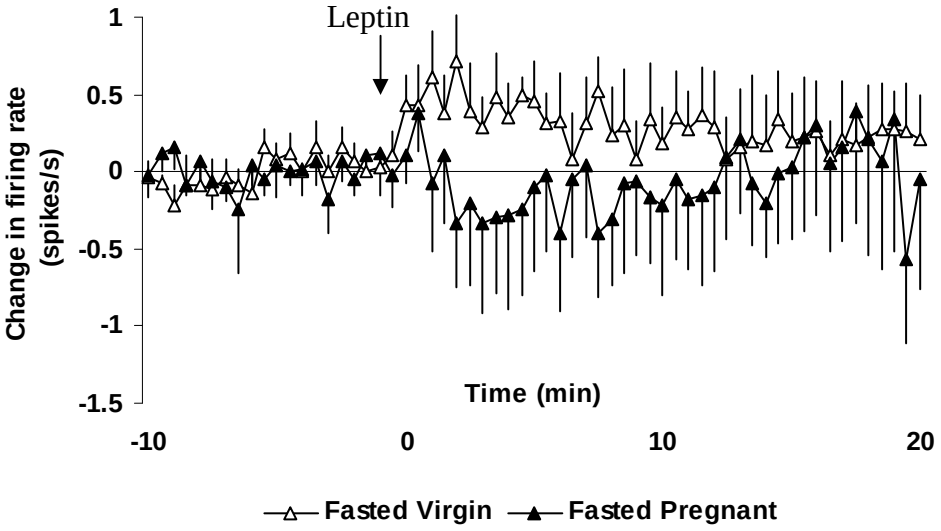

Fig. 4.16b. Effect of systemic administration of leptin (10µg; i.v) on electrical activity of SON oxytocin neurones in fasted virgin (n=6) and fasted pregnant (n=6) rats. Values are means ± s.e.m. of average changes from across 10 min of basal in 30 s bins. Pre- *vs.* 0-10min post-leptin within fasted virgin group: P=0.08, paired t-test. Pre- *vs.* 0-10min post-leptin within fasted pregnant group: P=0.7, paired t-test. 0-10min after leptin between fasted virgin and pregnant groups: P=0.3, t-test. [n.s]

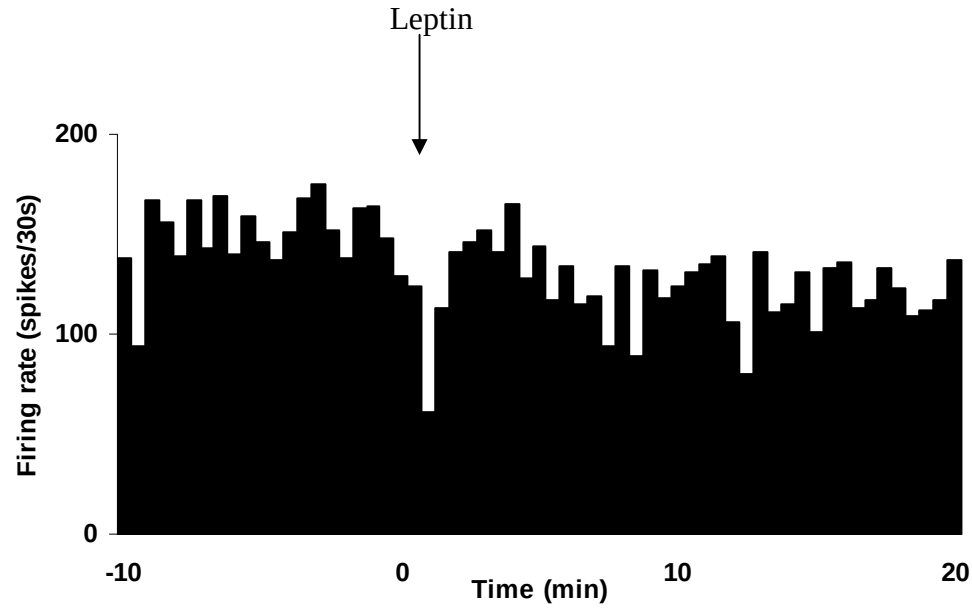

Fig. 4.17. Effect of systemic administration of leptin (100µg; i.v) on electrical activity of a non-phasic vasopressin neurone (Cell No. 214-1) in an unfasted virgin rat. The basal rate of 5 ± 0.1 spikes/s was decreased by 1.2 spikes/s 1min after leptin treatment.

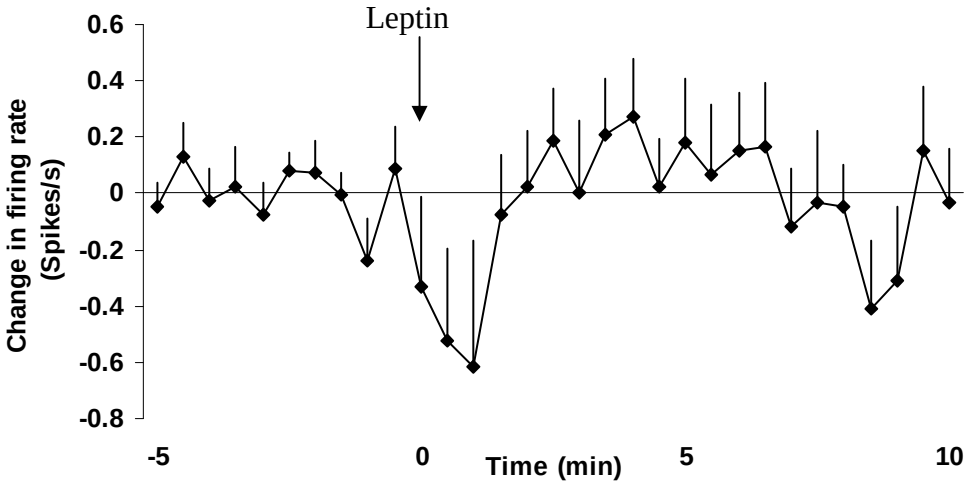

Fig. 4.18. Effect of systemic administration of leptin (100µg; i.v) on electrical activity of non-phasic vasopressin neurones in unfasted virgin rats (n=13). Values are means ± s.e.m. of average changes from across 10min of basal in 30s bins. Pre *vs.* 0-5min post-leptin: P=0.6, paired t-test. [n.s]

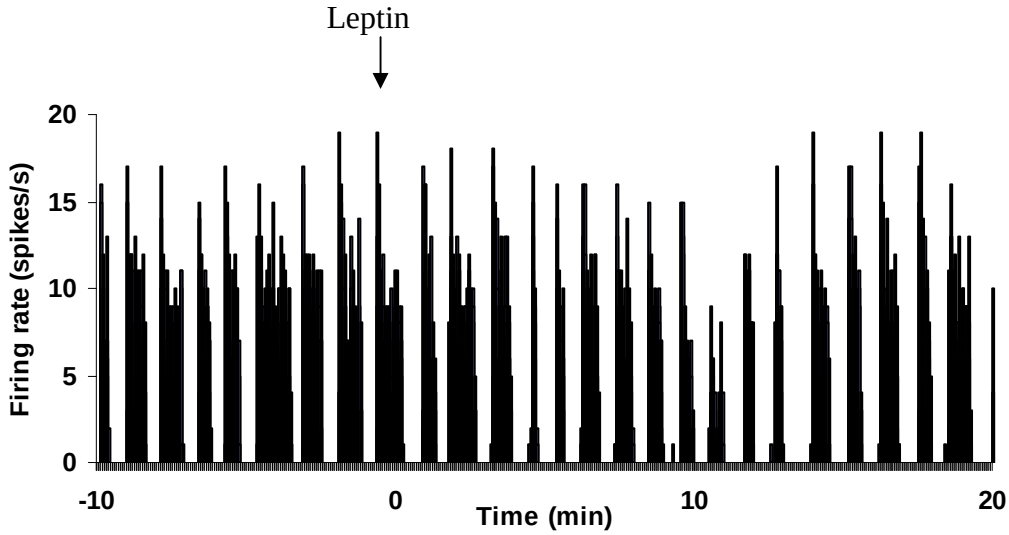

Fig. 4.19. Effect of systemic administration of leptin (100µg; i.v) on electrical activity of a phasic SON vasopressin neurone (Cell No. 220-1) in an unfasted virgin rat. Leptin elicited an inhibitory effect in this particular neurone. Comparing basal firing and firing 5-10min after leptin, the activity quotient decreased from 0.15 to 0.08, mean frequency within bursts decreased from 7.9 spikes/s to 7.8 spikes/s and mean interburst interval increased from 32.3s to 36.1s.

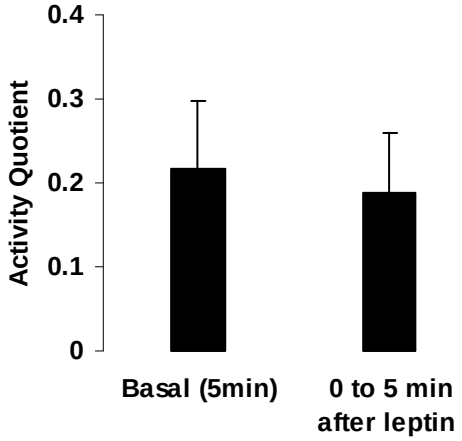

Fig. 4.20. Effect of systemic administration of leptin (100µg; i.v) on the electrical activity of phasic vasopressin neurones in unfasted virgin rats (n=8): Activity quotient. Values are mean ± s.e.m. Pre- *vs.* 0-5min post-leptin: P=0.3, paired t-test. [n.s]

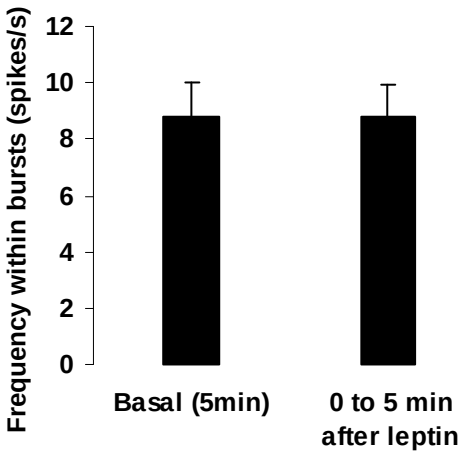

Fig. 4.21. Effect of systemic administration of leptin (100µg; i.v) on the electrical activity of phasic vasopressin neurones in unfasted virgin rats (n=8): Frequency within bursts (spikes/s). Values are mean ± s.e.m. Pre- *vs.* 0-5min post-leptin: P=0.85, paired t-test. [n.s]

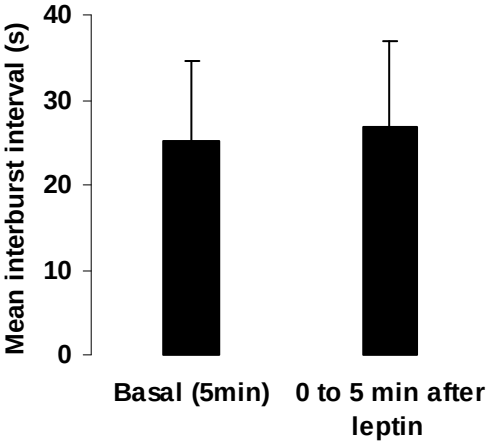

Fig. 4.22. Effect of systemic administration of leptin (100µg; i.v) on the electrical activity of phasic vasopressin neurones in unfasted virgin rats (n=8): Mean interburst interval (s). Values are mean ± s.e.m. Pre- *vs.* 0-5min post-leptin: P=0.7, paired t-test. [n.s]

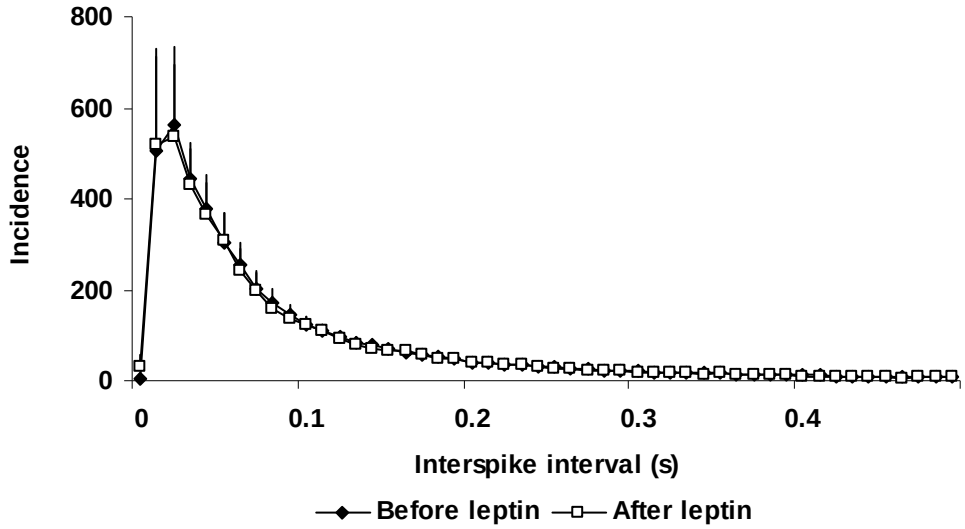

Fig. 4.23a. Effect of systemic administration of leptin (100µg; i.v) on the mean interspike interval of vasopressin neurones in unfasted virgin rats [n=19 (11 non-phasic and 8 phasic)]. Values are mean ± s.e.m; not normalised. There was no change in the histogram following leptin administration.

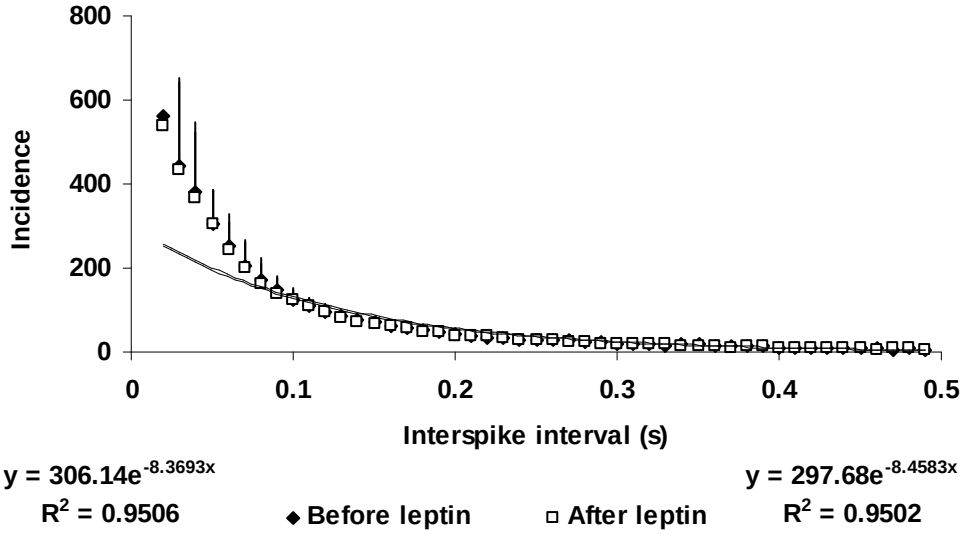

Fig. 4.23b. Effect of systemic administration of leptin (100µg; i.v) on the mean interspike interval of vasopressin neurones in unfasted virgin rats [n=19 (11 non-phasic and 8 phasic)]. Values are mean ± s.e.m. The descending slope of the histograms were fitted with exponential curves which fit only the distal tail of the histogram leaving intervals less than 0.2s unfit which is the characteristic of vasopressin neurones.

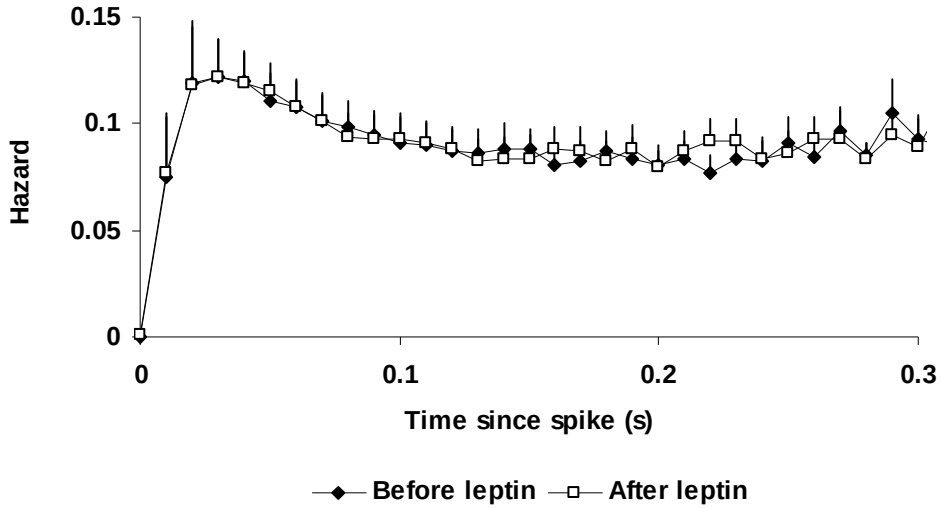

Fig. 4.24. Effect of systemic administration of leptin (100µg; i.v) on the mean post-spike probability of vasopressin neurones in unfasted virgin rats [n=19 (11 non-phasic and 8 phasic)]. Values are mean ± s.e.m; not normalised. There was no change in the shape of the hazard plot following leptin administration.

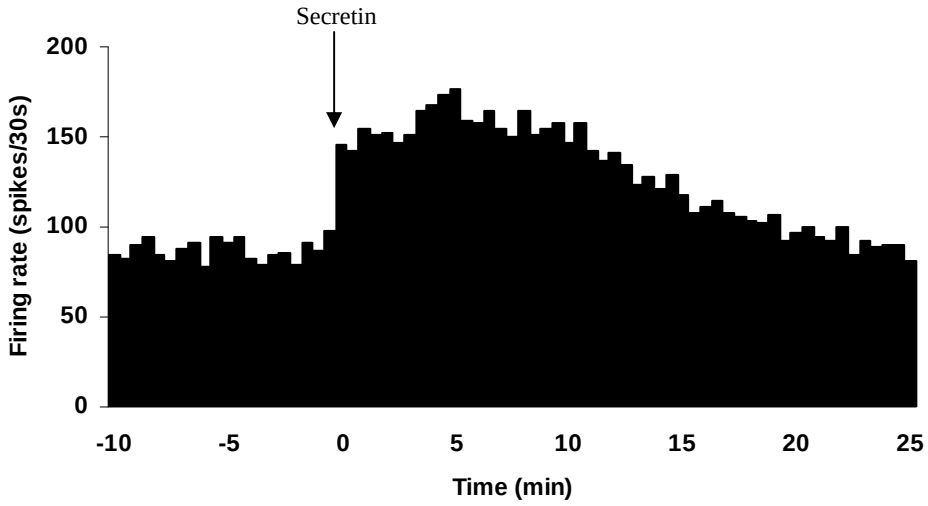

Fig. 5.1. Effect of systemic administration of secretin (0.1µg) on electrical activity of a SON oxytocin neurone (Cell No. 183-1) in an unfasted virgin rat. The basal firing rate of 2.9 ± 0.05 spikes/s was increased by ca. 3 spikes/s 5min after secretin. Firing rate gradually returned to basal rate again by 20min.

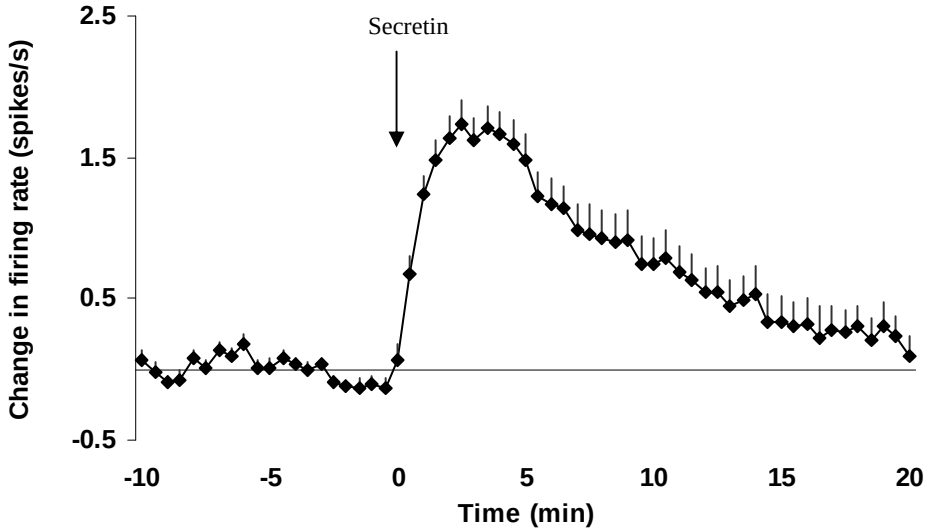

Fig. 5.2. Effect of systemic administration of secretin (0.1µg; i.v) on the firing rate of SON oxytocin neurones in unfasted virgin rats (n=26). Values are mean ± s.e.m. P<0.001, paired t-test, pre- *vs.* 0-10min post secretin. Change from mean basal, calculated over 10 min before i.v. secretin injection. Average basal firing rate: 4.1 ± 0.4 spikes/s. Systemic secretin, given at a near-physiological dose, significantly excited the SON oxytocin neurones.

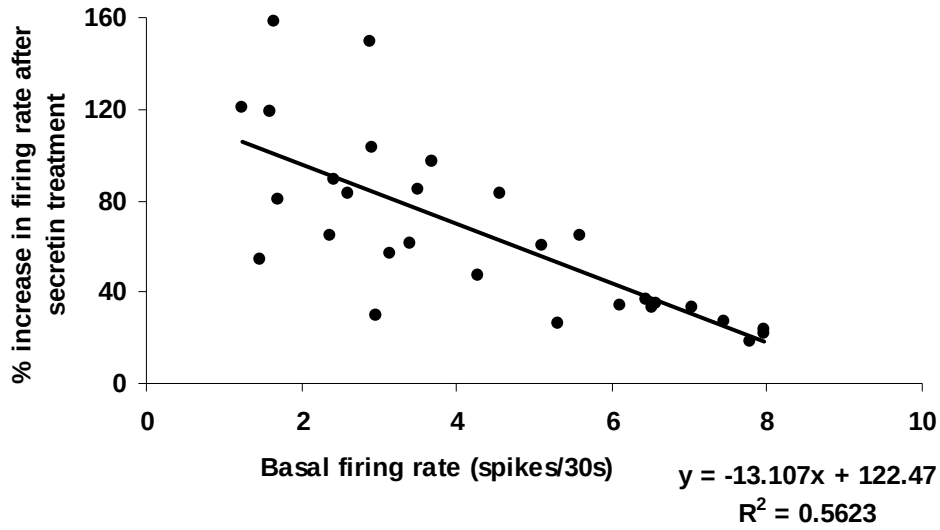

Fig. 5.2a. Correlation between the basal firing rate of SON oxytocin neurones and the percentage increase in firing rate after secretin (0.1µg/rat; i.v) administration in virgin rats. Basal firing rate and percentage increase in firing rate after secretin are negatively correlated ($P<0.001$; Pearson's correlation coefficient = -7.5).

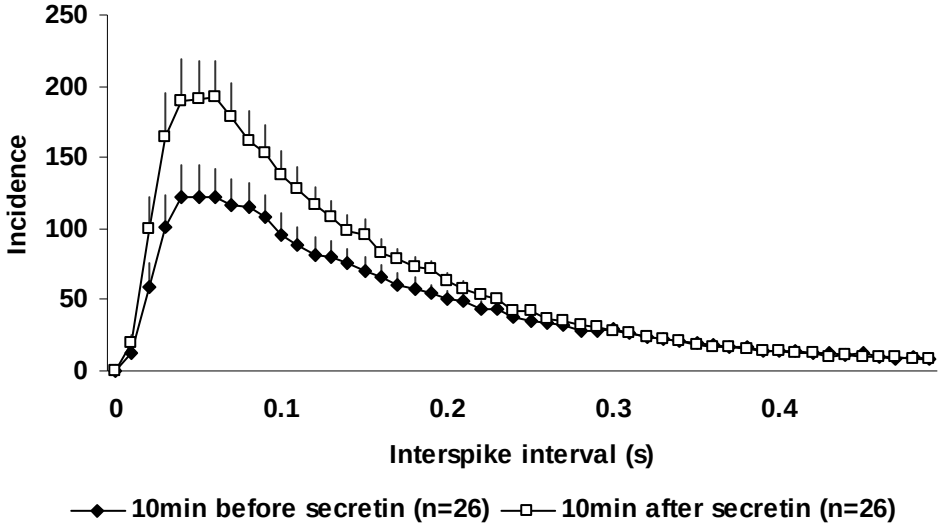

Fig. 5.3a. Effect of systemic administration of secretin (0.1µg; i.v) on the interspike interval histogram of SON oxytocin neurones in unfasted virgin rats. Values are mean ± s.e.m; not normalised. These interspike interval histograms 10min before and after the administration of secretin show that the number of spikes occurring within 0-0.5s after a spike is significantly increased after secretin. Pre- *vs.* post-secretin: P<0.001, paired t-test.

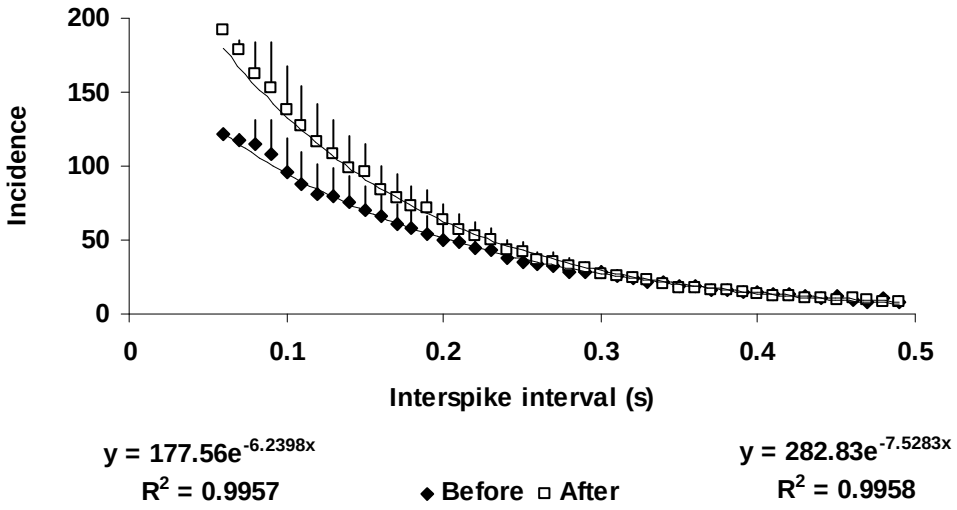

Fig. 5.3b. The interspike interval histograms fitted with exponential curves. Values are mean ± s.e.m. R^2 value of 0.99 indicates that the regression lines perfectly fit the data.

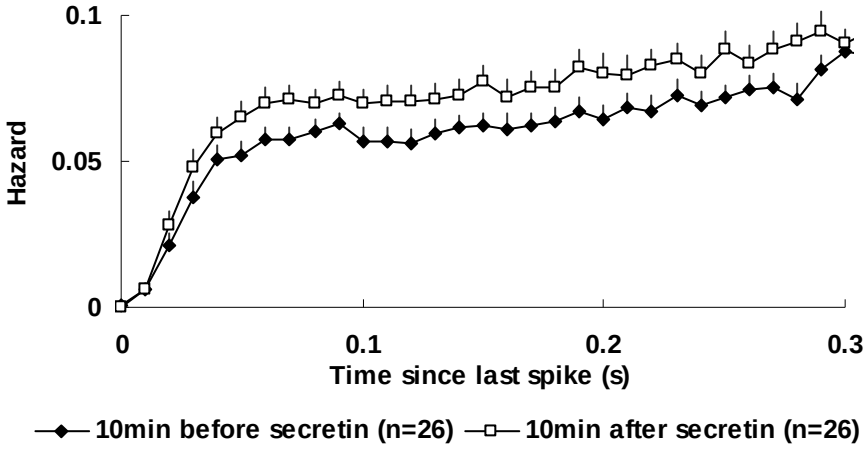

Fig. 5.4. Effect of systemic administration of secretin (0.1µg; i.v) on hazard analysis of SON oxytocin neurones in unfasted virgin rats. Values are mean ± s.e.m; not normalised. Pre- *vs.* post-secretin: P<0.001, Wilcoxon signed rank test. Secretin administration resulted in a positive shift in the hazard without a change in the shape of the hazard plot, suggesting that secretin-induced increase in the firing results from increase in the excitatory inputs not from change in the intrinsic properties of the neuronal membrane.

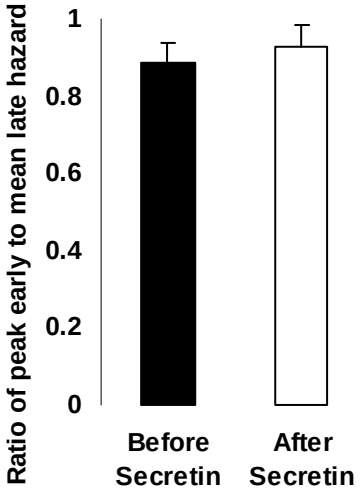

Fig. 5.5. Effect of systemic administration of secretin (0.1µg; i.v) on the ratio of peak early (<0.07s) to mean late (0.2-0.3s) hazard of SON oxytocin neurones in virgin rats (n=26). Values are mean ± s.e.m. Secretin administration did not alter the ratio of peak early to mean late hazard significantly (P=0.2, paired t-test) which confirms that there is no change in the shape of the hazard plot. [n.s]

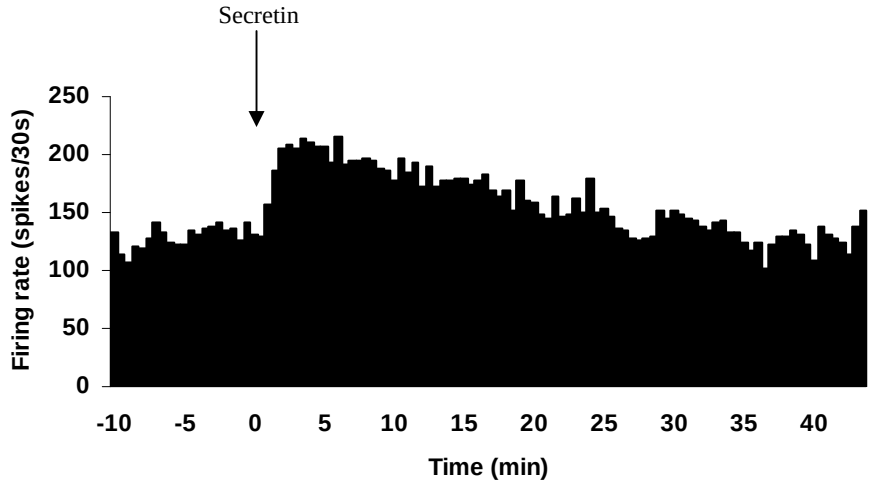

Fig. 5.6. Effect of systemic administration of secretin (0.1µg, i.v.) on the firing rate of a non-phasic SON vasopressin neurone in an unfasted virgin rat (Cell No. 235-2). Basal firing rate of 4.3 ± 0.07 spikes/s was increased by 2.6 spikes/s 3min after secretin. The rate returned to basal again by 25min.

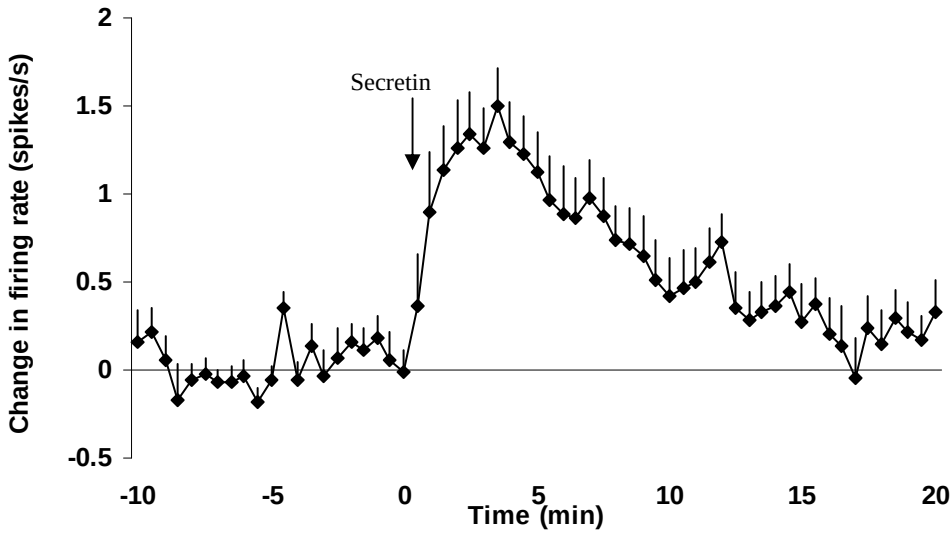

Fig. 5.7. Excitatory effects of systemic administration of secretin (0.1µg; i.v) on the electrical activity of non-phasic SON vasopressin neurones in unfasted virgin rats (n=14 out of 21). Values are mean ± s.e.m. Pre- vs 0-10min post-secretin: P=<0.001, paired t-test. Average basal firing rate: 5.8 ± 0.8 spikes/s.

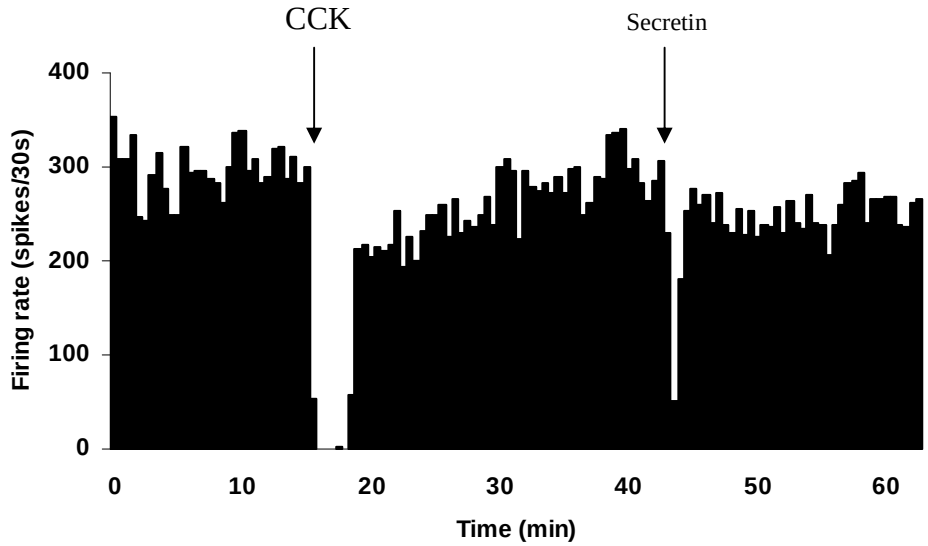

Fig. 5.8. Effect of systemic administration of secretin (0.1µg, i.v.) on firing rate of a non-phasic SON vasopressin neurone (245-1) in an unfasted virgin rat. CCK (25µg/kg; i.v) was given at 15 min. Basal rate before CCK was 9.9 ± 0.1 spikes/s and was decreased by 9.9 spikes/s 1 min after CCK. The rate remained low for 3min after CCK. Secretin (0.1µg; i.v) was given at 43 min. Firing rate before secretin injection was 9.7 ± 0.2 spikes/s and the rate was decreased by 8 spikes/s immediately after secretin. The rate remained low for 1min after secretin.

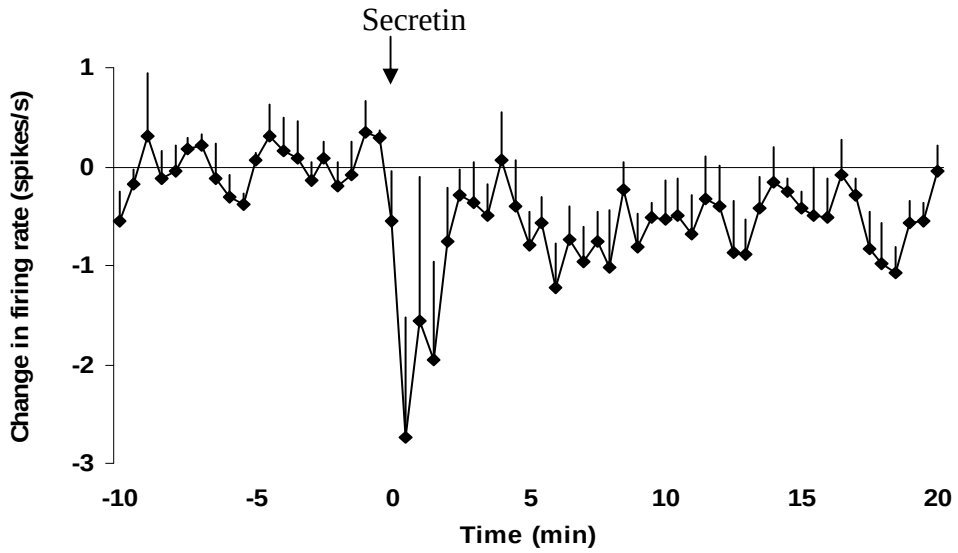

Fig. 5.9. Inhibitory effect of systemic administration of secretin (0.1µg; i.v) on firing rate of non-phasic SON vasopressin neurones in unfasted virgin rats (n=6 out of 21). Values are mean ± s.e.m. Pre- *vs.* 0-10min post-secretin: P=0.02, paired t-test. Average basal firing rate 7.2 ± 1.2 spikes/s.

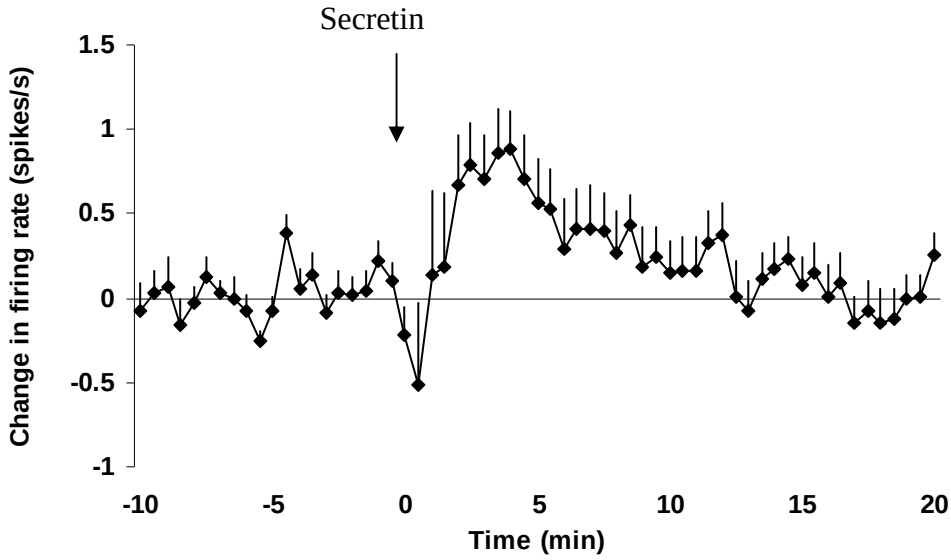

Fig. 5.10. Overall effect of systemic administration of secretin (0.1µg; i.v) on the firing rate of non-phasic SON vasopressin neurones in unfasted virgin rats (n=21). Values are mean ± s.e.m. Pre- *vs.* 0-10min post-secretin: P=0.2, paired t-test. Average basal firing rate: 6.3 ± 0.6 spikes/s. The effect of secretin on non-phasic SON vasopressin neurones was predominantly excitatory (i.e. 14 out of 21 neurones were excited); however, the overall response was not significant. [n.s]

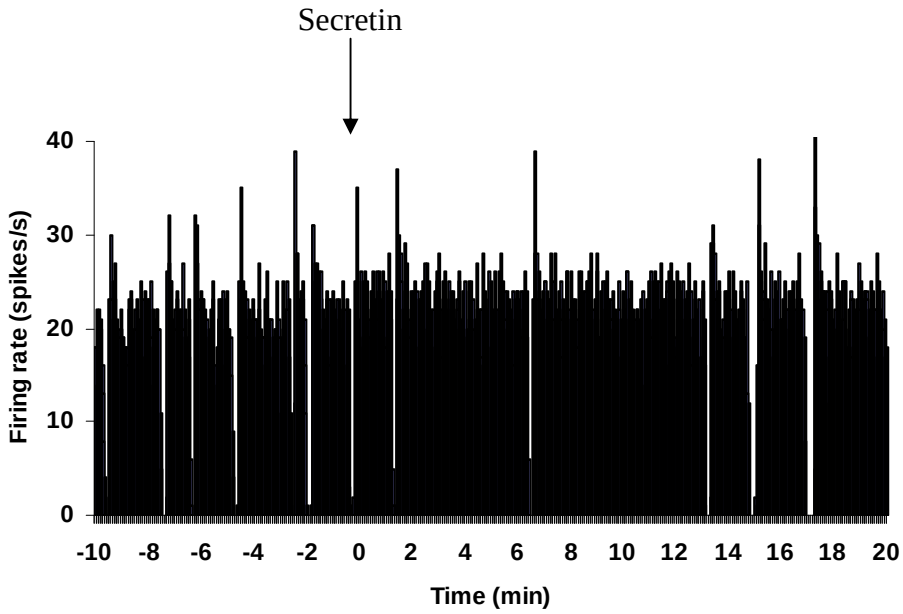

Fig. 5.11. Effect of systemic administration of secretin (0.1µg; i.v) on firing rate of a phasic SON vasopressin neurone in an unfasted virgin rat (Cell No. 246-3). Secretin (0.1µg; i.v) was given at 0min. Comparing the activity 5min before and 5min after secretin, the activity quotient increased from 0.2 to 0.5, frequency within bursts increased from 10.6 to 11.2 spikes/s and mean interburst time decreased from 11.7 to 8.8s.

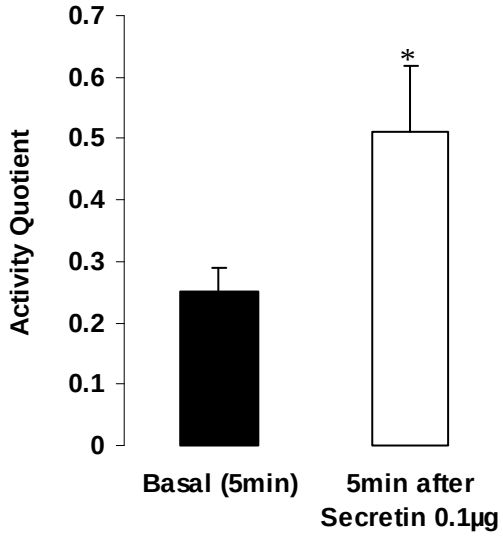

Fig. 5.12. Effect of systemic administration of secretin (0.1µg; i.v) on electrical activity of SON phasic vasopressin neurones in unfasted virgin rats (n=11): Activity Quotient. Values are mean ± s.e.m. *P=0.02, paired t-test, pre- *vs.* 0-5min post-secretin. Secretin administration significantly increased the activity quotient.

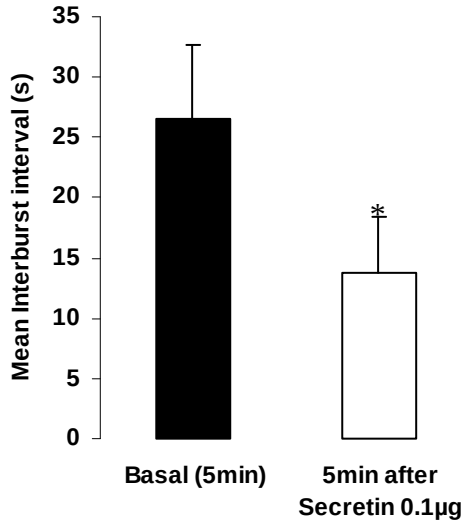

Fig. 5.13. Effect of systemic administration of secretin (0.1µg; i.v) on electrical activity of SON phasic vasopressin neurones in unfasted virgin rats (n=11): Mean interburst interval (s). Values are mean ± s.e.m. *P=0.014, Wilcoxon signed rank test, pre- *vs.* 0-5min post-secretin. Secretin administration significantly decreased the interburst interval and hence activated the phasic vasopressin neurones.

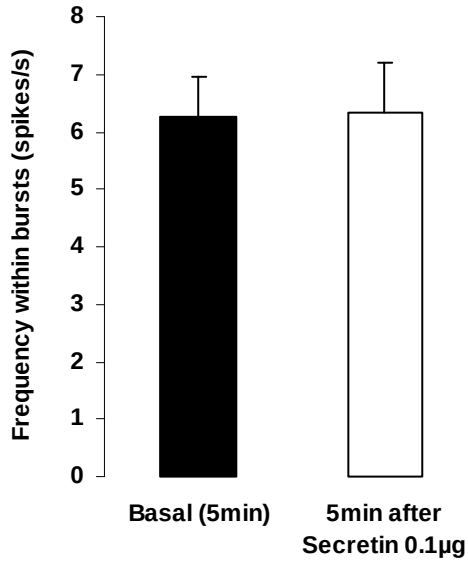

Fig. 5.14. Effect of systemic administration of secretin (0.1µg; i.v) on electrical activity of SON phasic vasopressin neurones in virgin rats (n=11): Frequency within bursts (spikes/s). Values are mean ± s.e.m. P=0.3, paired t-test, pre- *vs.*0-5min post-secretin. [n.s]

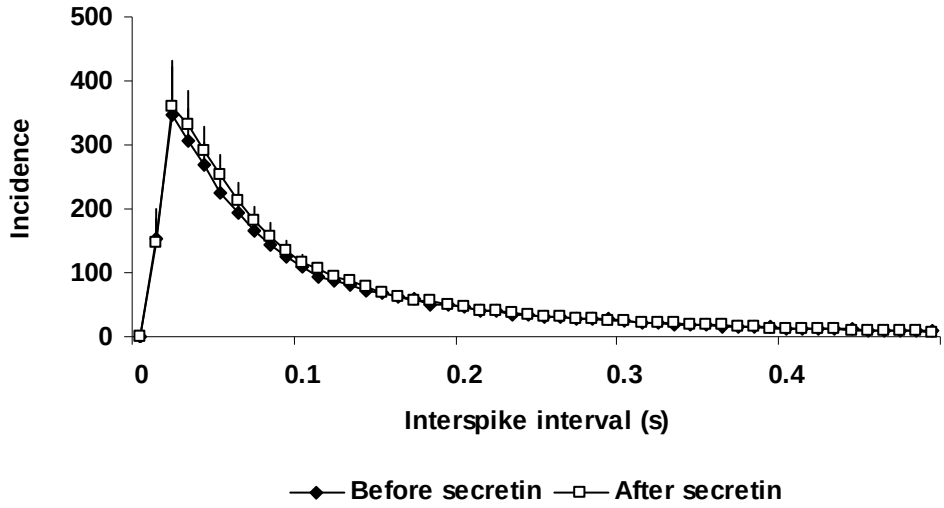

Fig. 5.15a. Effect of systemic administration of secretin (0.1µg; i.v) on interspike interval of SON vasopressin neurones in virgin rats (n=34; 21 non-phasic and 13 phasic vasopressin neurones). Values are mean ± s.e.m; not normalised. There was no change in the shape of the interspike interval histogram following secretin (P=0.2, paired t-test).

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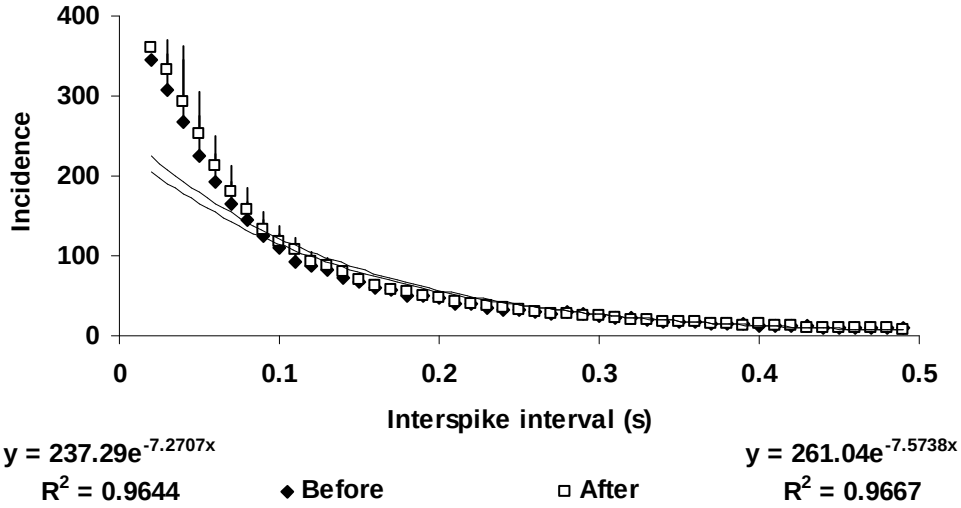


Fig. 5.15b. Interspike interval histograms of SON vasopressin neurones in virgin rats fitted with single negative exponential curves (n=34; 21 non-phasic and 13 phasic vasopressin neurones). Values are mean ± s.e.m. The negative exponential fits well with the distal tail of the histogram leaving the intervals less than 0.2s unfit. This is a characteristic of vasopressin neurones.

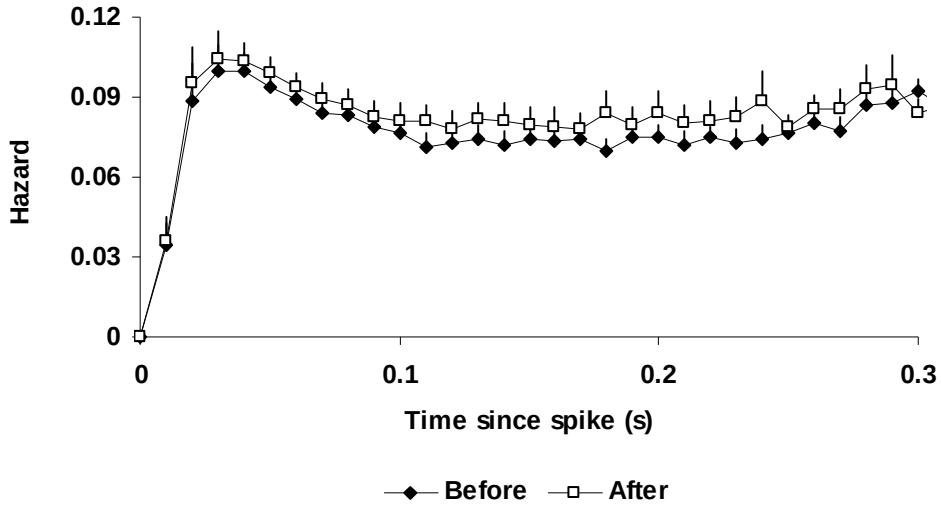


Fig. 5.16. Effect of systemic administration of secretin (0.1µg; i.v) on mean post-spike probability of SON vasopressin neurones in virgin rats (n=34; 21 non-phasic and 13 phasic vasopressin neurones). Values are mean ± s.e.m; not normalised. There was a significant positive shift in the hazard plot (P=0.001, Wilcoxon signed rank test) without obvious change in the shape of the plot following secretin.

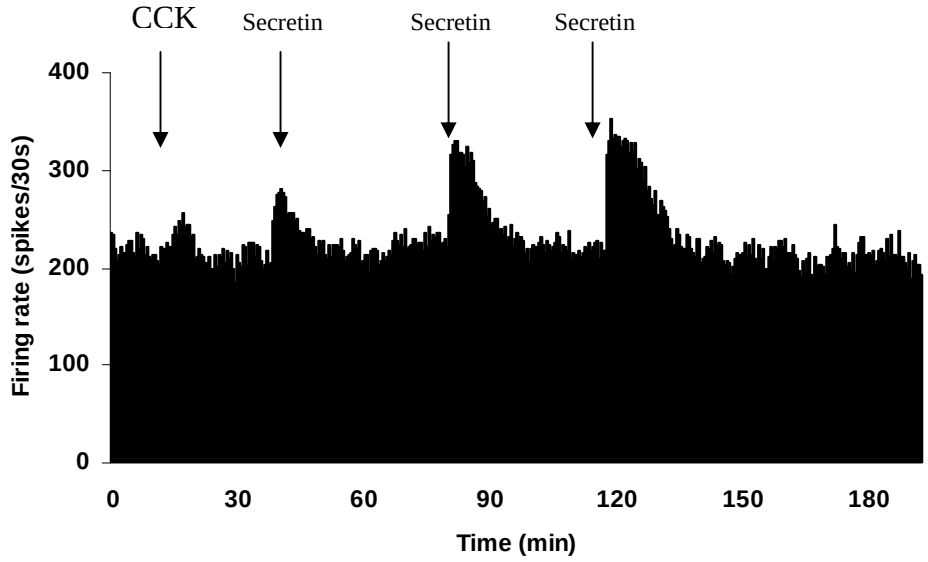


Fig. 5.17. Effects of systemic administration of increasing doses of secretin on a SON oxytocin neurone in a virgin rat (Cell No. 238-1). CCK (25µg/kg; i.v), secretin 0.1µg, 0.5µg and 1µg were given at 14, 38 80 and 117min, respectively. CCK briefly increased the firing rate. The firing rates before 0.1µg, 0.5µg and 1µgof secretin were 7 ± 0.09 spikes/s, 7.6 ± 0.05 spikes/s and 7.3 ± 0.05 spikes/s, respectively, and were increased by 2.3 spikes/s by 2.5min, 3.4 spikes/s by 2min and 4.4 spikes/s by 1.5min, respectively after secretin.

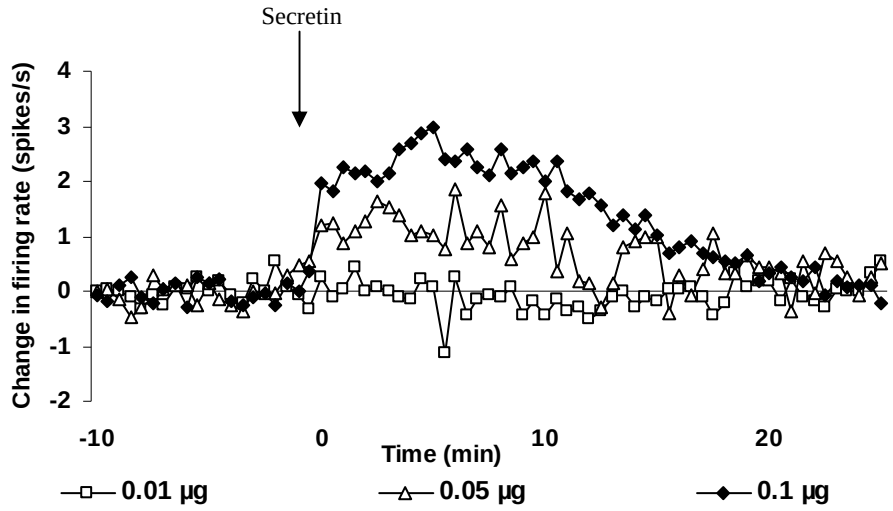


Fig. 5.18. Effects of systemic administration of increasing doses of secretin on firing rate of a SON oxytocin neurone (Cell No. 183-1) in a virgin rat. The basal firing rates before 0.01µg, 0.05 µg and 0.1 µg secretin were 2.5 ± 0.05spikes/s, 2.5 ± 0.06 spikes/s and 2.9 ± 0.05 spikes/s, respectively. 0.01µg of secretin did not affect the firing rate while the rate was increased by 1.65 spikes/s by 2.5min and 3 spikes/s by 5min after 0.05 µg and 0.1 µg of secretin, respectively.

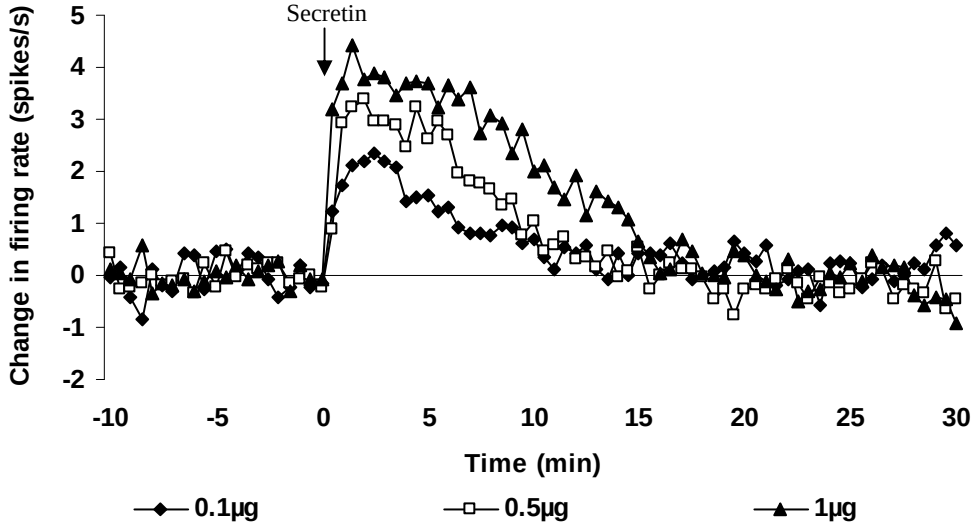


Fig. 5.19. Effects of increasing doses of secretin on firing rate of a SON oxytocin neurone (No. 238-1) in a virgin rat. The firing rates before 0.1µg, 0.5 µg and 1 µg secretin were 7 ± 0.09 spikes/s, 7.6 ± 0.05 spikes/s and 7.3 ± 0.05 spikes/s, respectively. The rate was increased by 2.3 spikes/s by 2.5min, 3.4 spikes/s by 2min and 4.4 spikes/s by 1.5min respectively after 0.1µg, 0.5 µg and 1 µg of secretin.

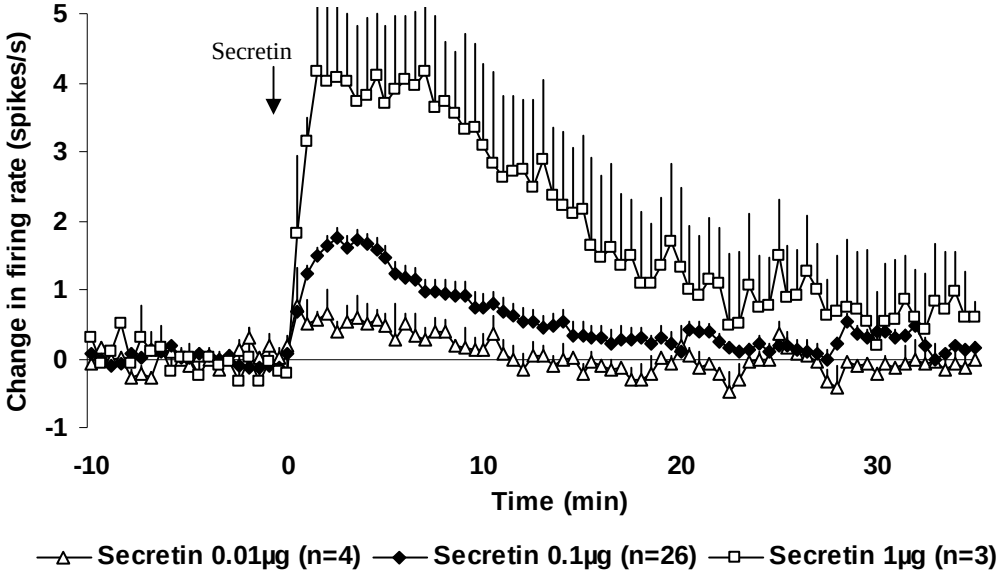


Fig. 5.20. Effects of systemic administration of different doses of secretin on SON oxytocin neurones in virgin female rats. Values are means ± s.e.m. Group numbers are: 1µg: n=3; 0.1µg: n=26; 0.01µg: n=4. P=0.04, paired t-test, pre- *vs.* 0-10min post-secretin within the 1µg group; P<0.001, paired t-test, pre- *vs.* 0-10min post-secretin within the 0.1µg group. 0-10min post-secretin between the groups: P<0.001, one-way ANOVA. Secretin-induced excitatory responses in SON oxytocin neurones were dose dependent.

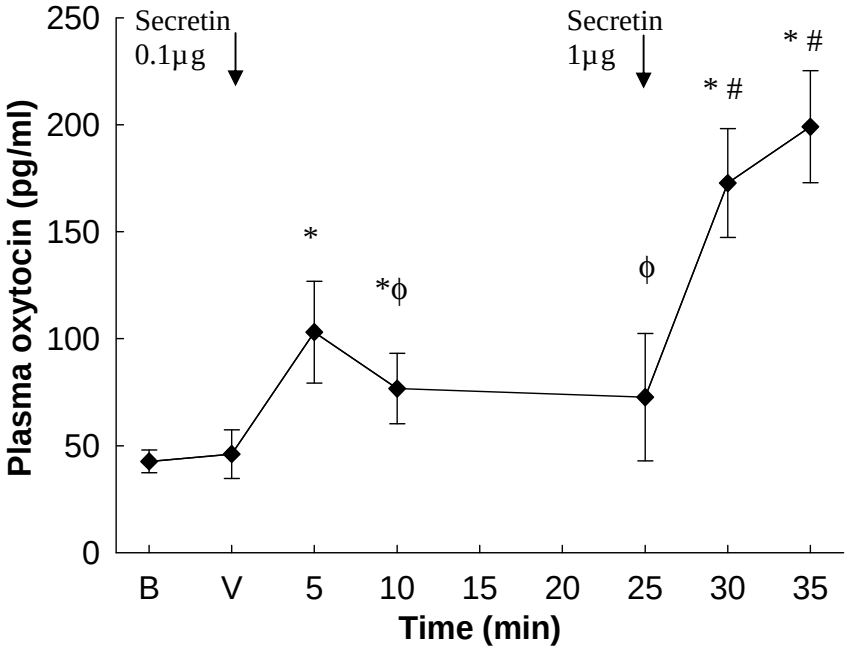


Fig. 5.21. Effect of systemic administration of secretin (0.1 and 1µg/rat; i.v; n=6) on the plasma oxytocin secretion in urethane anaesthetized unfasted rats. Values are mean ± s.e.m. Basal sample (B) was collected 2h after the cannulation surgery; a sample (V) was taken 5min after vehicle (100µl normal saline; i.v). Subsequently, samples were collected 5, 10 and 25min after administration of 0.1µg secretin and 5 and 10min after 1µg secretin. * $P<0.05$ (0.1µg secretin) and $P=0.001$ (1µg secretin), one-way RM ANOVA, *vs.* basal and vehicle control. ϕ $P<0.05$, one-way RM ANOVA, *vs.* 5 min after 0.1µg secretin. # $P=0.001$, one-way RM ANOVA, *vs.* 5 and 10min after 0.1µg secretin, at respective time points. Systemic secretin dose-dependently increased plasma oxytocin release.

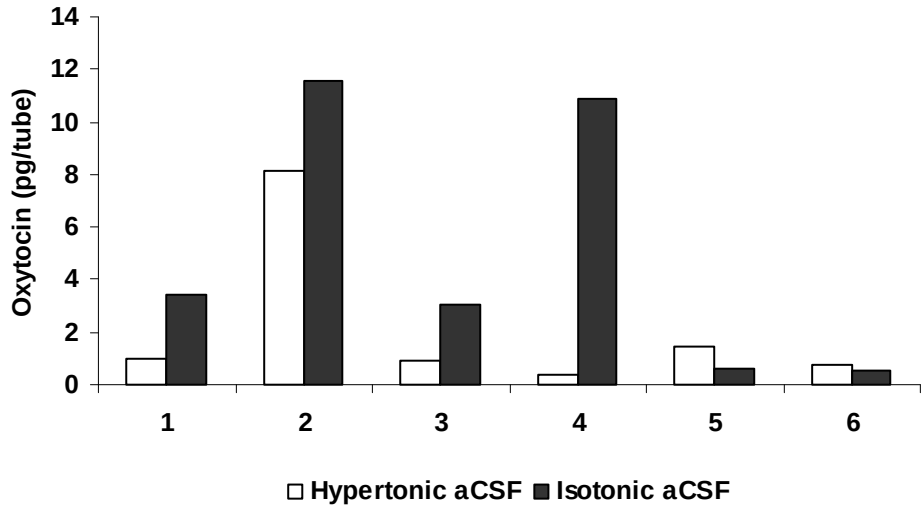


Fig. 5.22a. Effect of microdialysis of hypertonic aCSF on somato-dendritic oxytocin release from the SON in vivo in four urethane-anaesthetized female rats (No. 1-6). Hypertonic aCSF (1M NaCl) was microdialysed at the end of the experiment followed by isotonic aCSF. Four out of six rats (No. 1-4) showed a positive response to hypertonic and subsequent aCSF microdialysis and were statistically analysed to seek evidence of secretin-induced central oxytocin release from the SON.

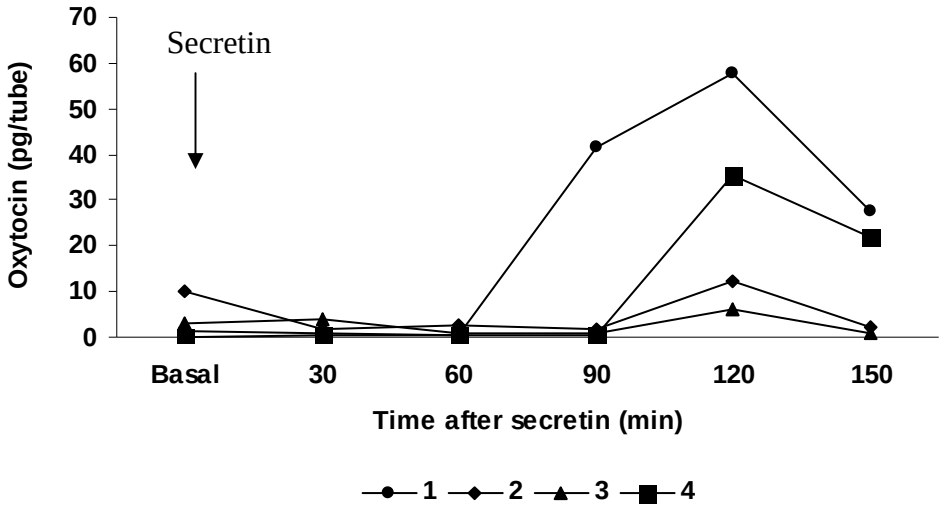


Fig. 5.22b. Effect of systemic administration of secretin (1µg/rat, i.v.) on central oxytocin release *in vivo* in four urethane-anaesthetized female rats (No. 1-6). Secretin was administered immediately after collecting the basal sample. Four out of six rats showed a delayed excitatory response to systemic secretin (1µg/rat) more than an hour after its administration.

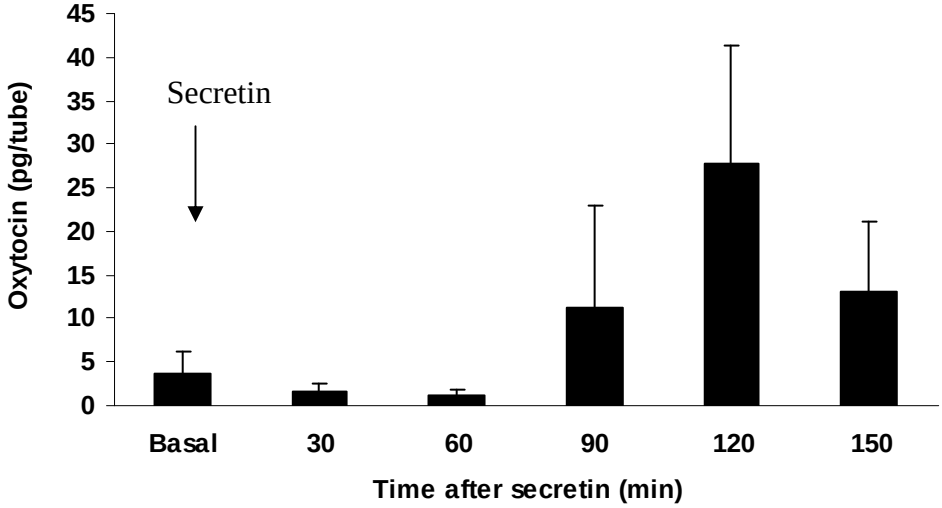


Fig. 5.22c. Effect of systemic administration of secretin (1µg/rat) on somato-dendritic oxytocin release from the SON *in vivo* in urethane-anaesthetized female rats (n=4). Values are mean ± s.e.m. Secretin was administered immediately after collecting the basal sample. Data are for the four out of six rats that showed excitatory response to hyperosmotic stimulation. The excitatory response after secretin approached significance (F=2.7, P=0.06, one-way RM ANOVA).

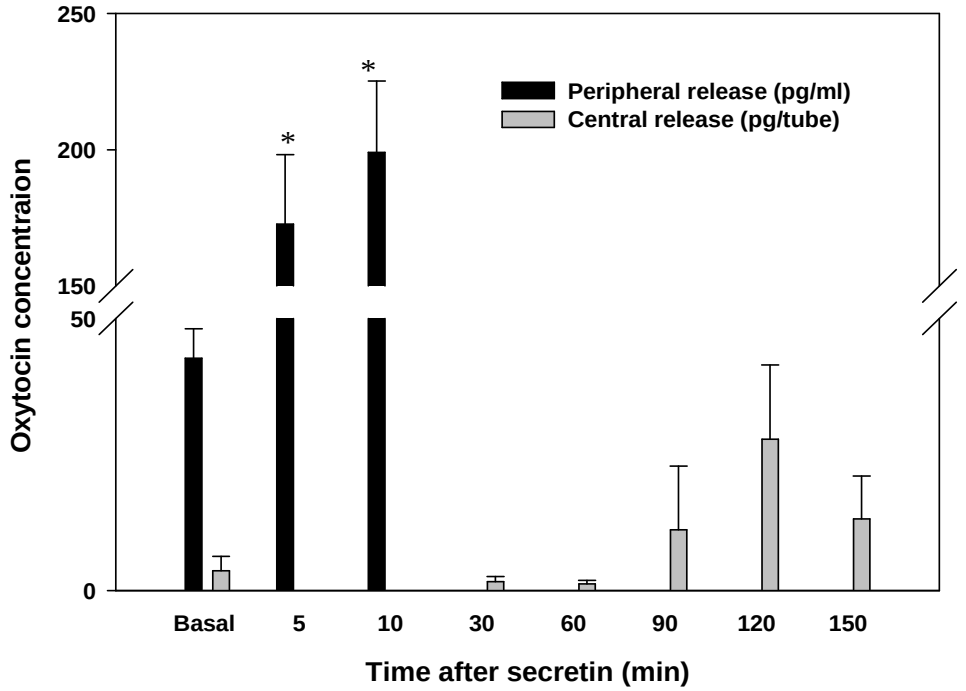


Fig. 5.22d. Effect of systemic administration of secretin (1µg/rat) on peripheral (n=6; data from Fig. 5.21) and SON (n=4; data from Fig 5.22c) oxytocin release *in vivo* in urethane-anaesthetized female rats. Values are mean ± s.e.m. Systemic secretin-induced peripheral oxytocin release was immediate while SON release was delayed. Increase in peripheral oxytocin release following secretin was significant (* *vs.* basal, $F=39.8$, $P<0.001$, one-way RM ANOVA followed by Bonferroni's pairwise multiple comparison procedure) while SON oxytocin release approached significance ($F=2.7$, $P=0.06$, one-way RM ANOVA).

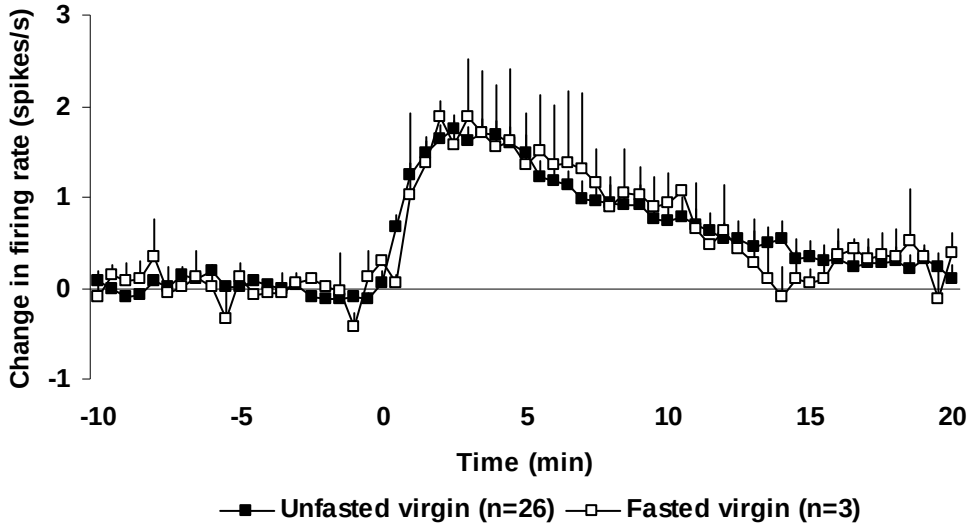


Fig. 5.23. Comparison of the effects of systemic administration of secretin (0.1µg; i.v) on the electrical activity of SON oxytocin neurones in fasted with unfasted rats. Values are mean ± s.e.m. P=0.05, paired t-test, pre- *vs.* 0-10min post-secretin within fasted group. P<0.001, paired t-test, pre *vs.* 0-10min post-secretin within unfasted group. Fasted vs unfasted rats: 0-10min after secretin: P=0.9, t-test. Secretin-induced change in the firing rate of SON oxytocin neurones in fasted virgin rats was not different from unfasted virgin rats.

Fig. 5.24. Effect of systemic administration of secretin (0.1µg; i.v) on the electrical activity of phasic firing SON vasopressin neurones in fasted and unfasted rats: Activity quotient. Values are mean ± s.e.m. *P=0.02, paired t-test, pre- *vs.* 0-5min post-secretin within unfasted rats. Pre- *vs.* post-secretin in fasted rats: P=0.1, paired t-test. Fasted *vs.* unfasted rats: basal: P=0.3, t-test; post-secretin: P=0.2, t-test. There was no difference between fasted and unfasted rats in basal and secretin-induced activity of phasic firing SON vasopressin neurones.

Fig. 5.25. Effect of systemic administration of secretin (0.1µg; i.v) on the electrical activity of phasic firing SON vasopressin neurones in fasted and unfasted rats: Mean interburst interval (s). Values are mean ± s.e.m. *P= 0.014, Wilcoxon signed rank test, pre- *vs*.0-5min post-secretin within unfasted rats. Pre- *vs*. post-secretin in fasted rats: P= 0.4, paired t-test. Fasted *vs*. unfasted rats: basal: P=0.97, t-test; post-secretin: P=0.4, t-test. There was no difference between fasted and unfasted rats in basal and secretin-induced activity of phasic firing SON vasopressin neurones.

Fig. 5.26. Effect of systemic administration of secretin (0.1µg; i.v) on the electrical activity of phasic firing SON vasopressin neurones in fasted (n=3) and unfasted (n=11) virgin rats: Frequency within bursts (spikes/s). Values are mean ± s.e.m. Pre- *vs*. post-secretin in unfasted rats: P=0.3, paired t-test. Pre- *vs*. post-secretin in fasted rats: P=0.3, paired t-test. Fasted *vs.* unfasted rats: basal: P=0.5, t-test; post-secretin: P=0.8, t-test. There was no difference between fasted and unfasted rats in basal and secretin-induced activity of phasic firing SON vasopressin neurones. [n.s]

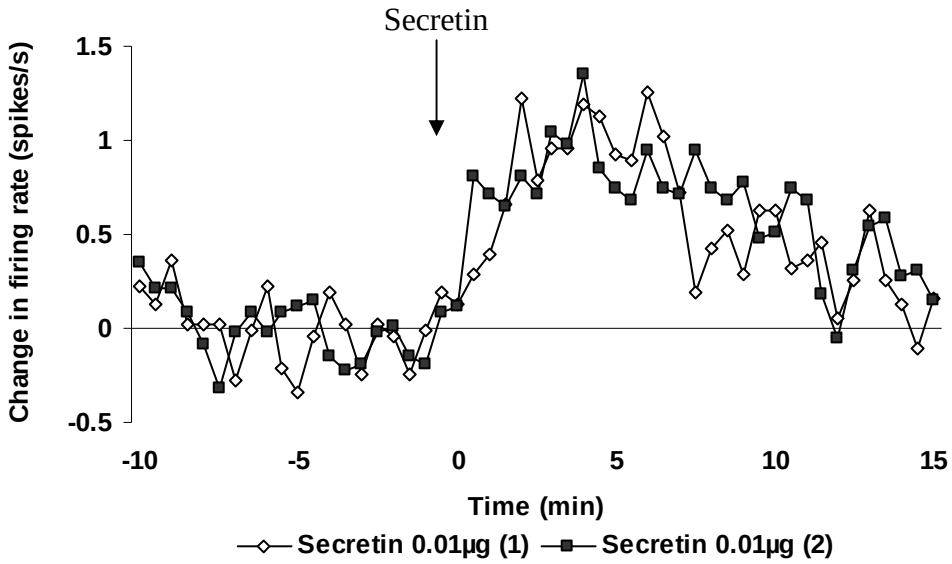


Fig. 5.27. Excitatory responses to repeated injections of secretin (0.01µg; i.v) in a SON oxytocin neurone in an unfasted rat (Cell No. 202-3). The average basal firing rates before secretin treatments were 3.3 ± 0.04 spikes/s and 2.9 ± 0.04 spikes/s. These were increased by 1.2 spikes/s 2min after the first secretin injection and by 1.3 spikes/s 4min after the second secretin injection. The responses gradually returned to basal rate by 15min after secretin. The responses to repeated injections of secretin were not different.

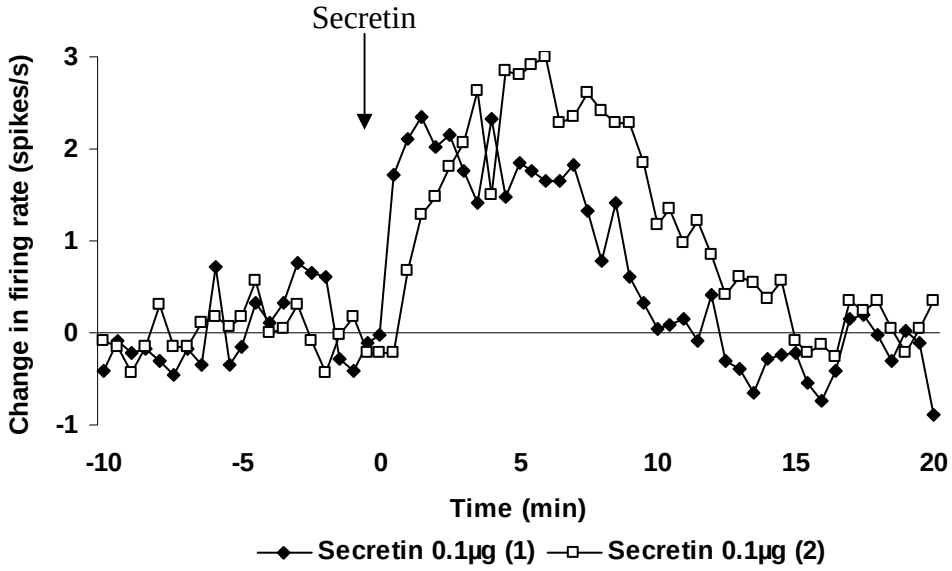


Fig. 5.28. Excitatory responses to repeated injections of secretin (0.1µg; i.v) in a SON oxytocin neurone in a virgin rat (Cell No. 266-2). The average basal firing rates before secretin treatment were 5.4 ± 0.09 spikes/s and 4.8 ± 0.06 spikes/s. These were increased by 2.4 spikes/s 1.5min after the first secretin injection and by 3.0 spikes/s 6min after the second secretin injection. The responses gradually returned to basal rate by 10-15min after secretin. The responses to repeated injections of secretin were not different.

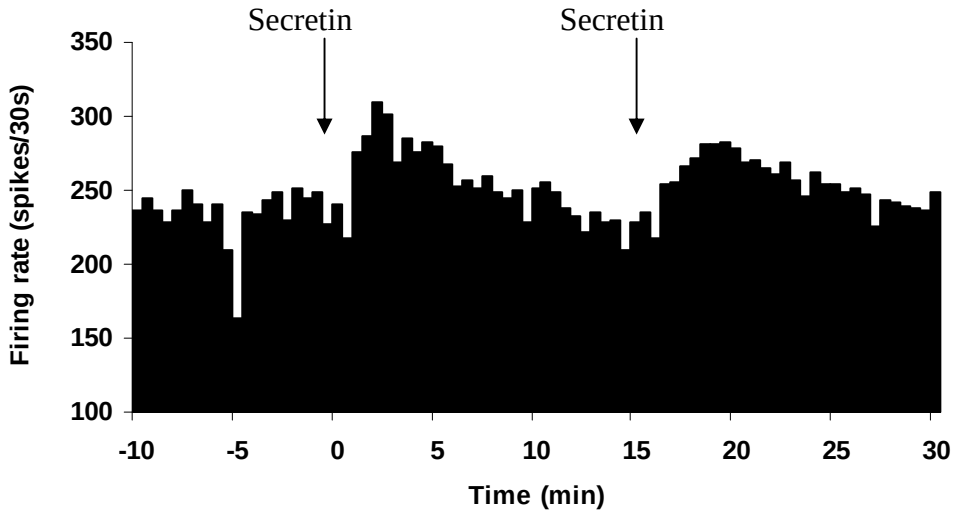


Fig. 5.28a. Excitatory responses to repeated injections of secretin (0.1µg; i.v) in a SON oxytocin neurone in a virgin rat (Cell No. 273-3). Secretin was given at 0 and 15min.

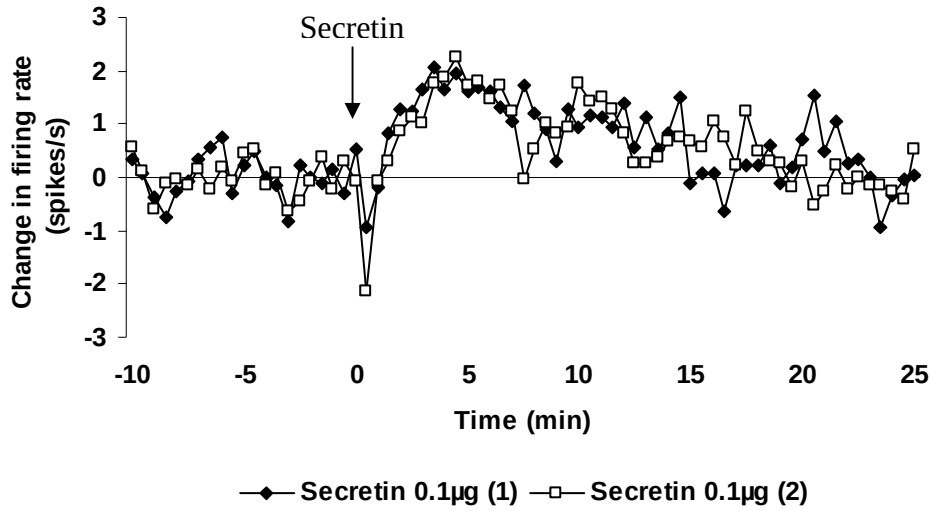


Fig. 5.29. Excitatory responses to repeated injections of secretin (0.1µg; i.v) in a SON vasopressin neurone in a virgin rat (Cell No. 267-1). The average basal firing rates before secretin treatments were 4.7 ± 0.09 spikes/s and 4.3 ± 0.08 spikes/s. These were increased by 2.05 spikes/s 3.5min after the first secretin injection and by 2.2 spikes/s 4.5min after the second secretin injection. The responses gradually returned to basal rate by 15min after secretin. The responses to repeated injections of secretin were not different.

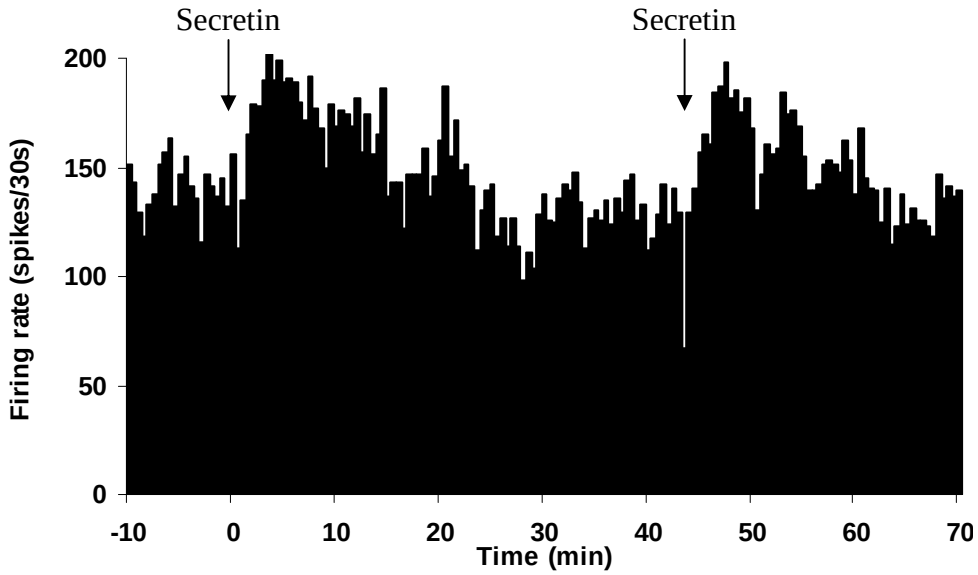


Fig. 5.29a. Excitatory responses to repeated injections of secretin (0.1µg; i.v) in a SON vasopressin neurone in a virgin rat (Cell No. 267-1). Secretin was given at 0 and 43rd min.

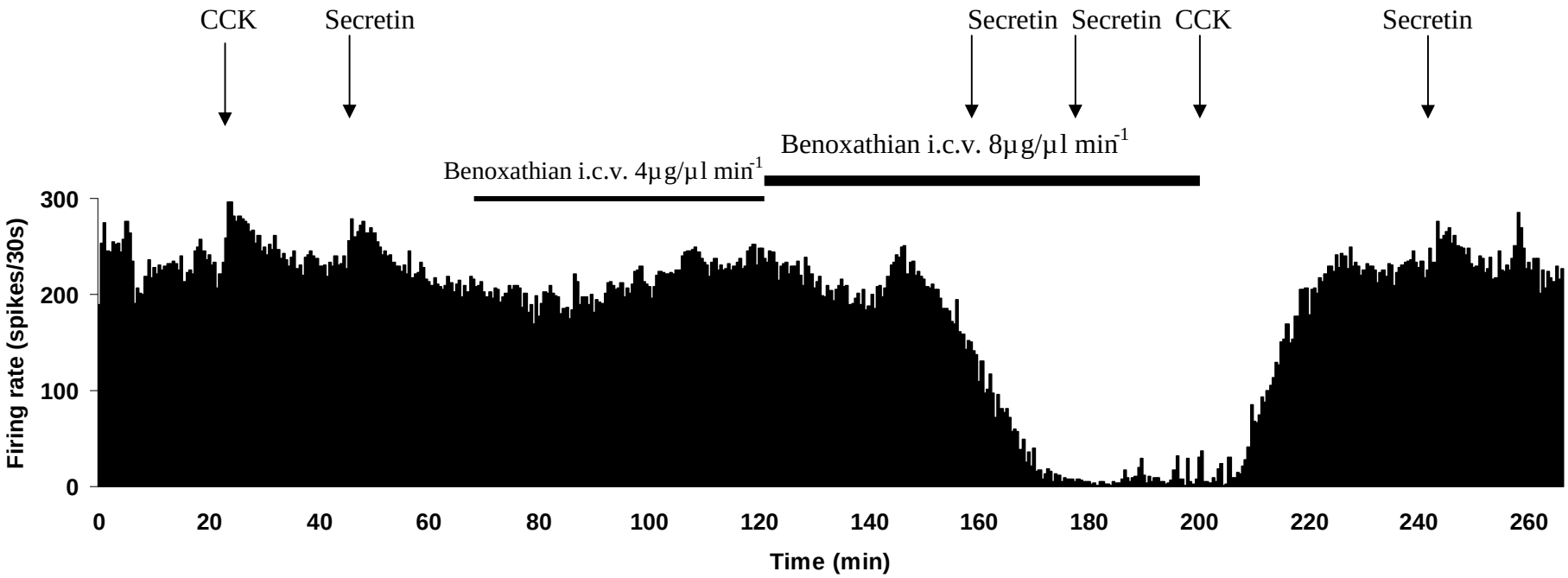


Fig. 5.30. Effect of i.c.v. benoxathian infusion on secretin-induced excitatory firing rate response of a SON oxytocin neurone in a virgin rat (Cell No. 258-1). CCK (25µg/kg; i.v) was given at 23min (before benoxathian i.c.v. infusion) and 200min (73.5 min after starting higher dose of benoxathian). Secretin (0.1µg; i.v) was given 45.5min (before benoxathian i.c.v. infusion), 162min (35.5min after starting higher dose of benoxathian) 178.5th (52 min after starting higher dose of benoxathian) and 243min. Benoxathian i.c.v. infusion (4µg/0.5µl min^{-1}) was started at 66.5min. The dose rate was increased to 8µg/1µl min^{-1} from 126.5min as the lower dose did not reduce the basal rate. I.c.v. benoxathian infusion was stopped at 203.5min. Basal firing rate before starting the higher dose of benoxathian was 7.87±0.08 spikes/s and was decreased by 4.47 spikes/s 35min after starting benoxathian. I.c.v. Benoxathian infusion blocked the secretin-induced excitatory response in this neurone. The response returned after stopping the infusion. [Note: The basal rate of only two out of the six cells tested was suppressed by benoxathian].

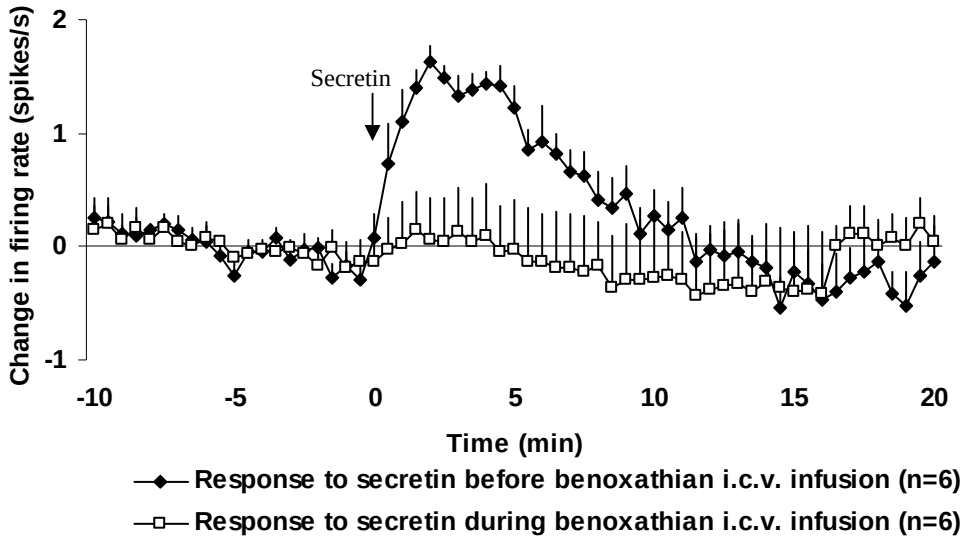


Fig. 5.31. Effect of i.c.v. benoxathian infusion on secretin (0.1µg; i.v)-induced excitatory firing rate response of SON oxytocin neurones in unfasted virgin rats. Values are mean ± s.e.m. Pre- *vs.* 0-10min post-secretin before benoxathian: P<0.001, paired t-test. 0-5min post-secretin before *vs.* during benoxathian: P=0.006, t-test. Pre- *vs.* 0-10min post-secretin during benoxathian: P=0.7, Wilcoxon signed rank test. Basal firing rates before secretin before and during benoxathian i.c.v. infusion were 5.65 ± 1.05 spikes/s and 2.2 ± 0.9 spikes/s, respectively. Secretin-induced excitation of SON oxytocin neurones was significantly decreased by benoxathian i.c.v. infusion.

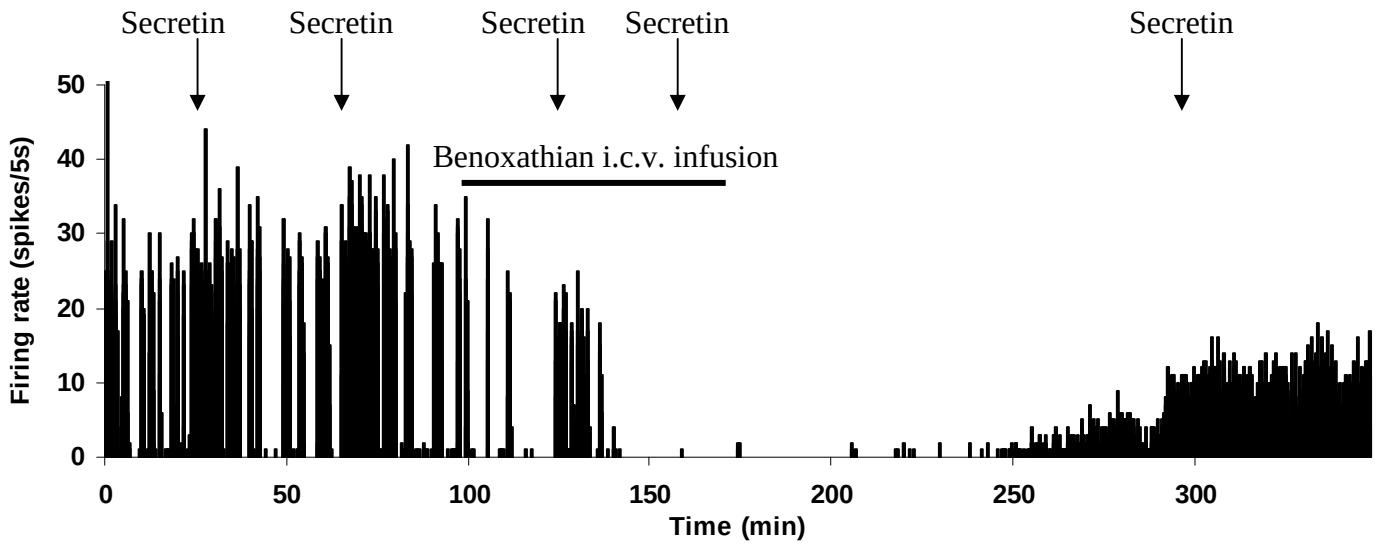


Fig. 5.32. Effect of benoxathian i.c.v. infusion on secretin-induced excitatory response in a phasic firing SON vasopressin neurone (Cell No. 272-1). Benoxathian infusion (8µg/µl; min^{-1}; i.c.v) was started at 100min and stopped at 171min. Secretin (0.1µg; i.v) was given at (1) 23min (before benoxathian infusion), (2) 67min (before benoxathian infusion), (3) 123min (23min after starting benoxathian infusion), (4) 159min (58min after starting benoxathian infusion) and at (5) 291min (120min after stopping benoxathian infusion). Secretin given before benoxathian infusion at 23 and 67min increased the activity quotient [secretin (1): from 0.07 to 0.38; secretin (2): from 0.28 to 0.43]. Secretin (3) given 23min after starting benoxathian infusion elicited an excitatory response (activity quotient increased from 0 to 0.3) probably because it was given too early that benoxathian had not exerted its effect yet. Benoxathian infusion totally blocked the basal activity and secretin (4) given 58min after starting benoxathian infusion did not induce any activity. The cell started to regain its basal activity from 30min after stopping benoxathian infusion. Secretin (5) given 120min after stopping the infusion elicited a mild excitatory response (activity quotient increased from 0.03 to 0.18).

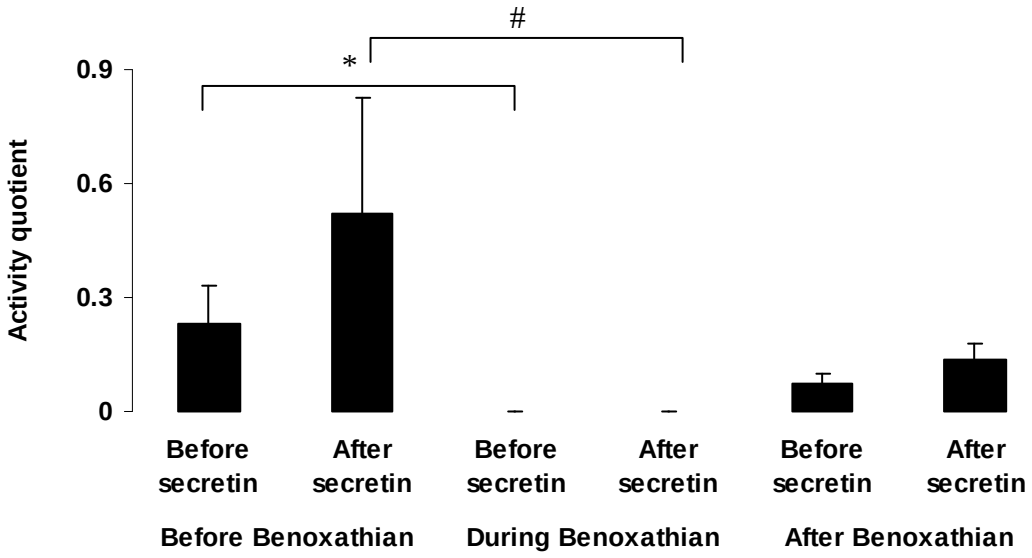


Fig. 5.33. The effect of benoxathian i.c.v. infusion on the basal and secretin-induced activity of phasic firing SON vasopressin neurones in unfasted rats (n=3): Activity quotient. Values are mean ± s.e.m. * P=0.04, one-way ANOVA, basal activity before *vs.* during benoxathian infusion; # P=0.03, one-way ANOVA, secretin-induced activity before *vs.* during benoxathian infusion. Benoxathian i.c.v. infusion completely blocked the basal activity and secretin-induced increase in activity of SON vasopressin neurones.

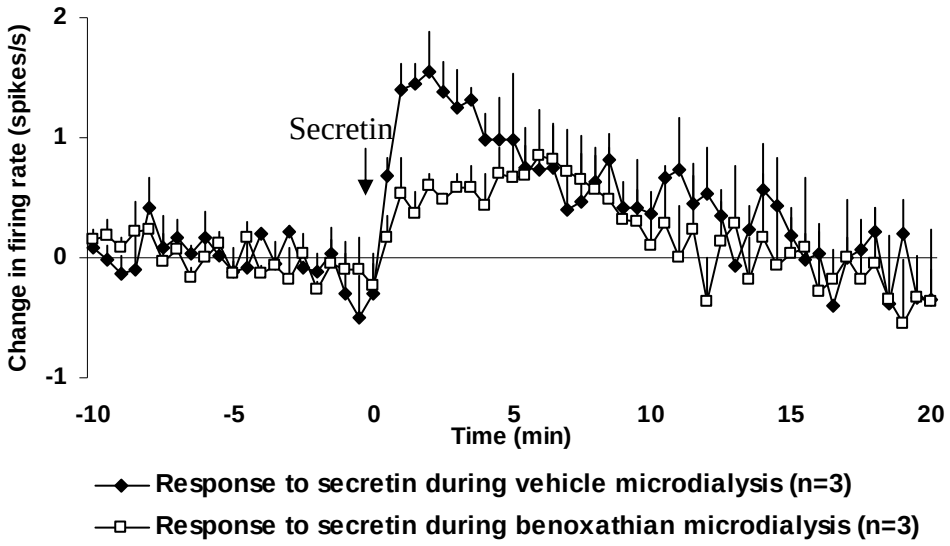


Fig. 5.34. Effect of benoxathian (2mM) microdialysis onto the SON on secretin-induced (0.1µg; i.v) excitatory response of SON oxytocin neurones in unfasted female rats. Values are mean ± s.e.m. Secretin-induced excitatory response during vehicle microdialysis *vs.* during benoxathian microdialysis: P=0.03; t-test. Pre- *vs.* post-secretin during vehicle microdialysis: P=0.03, paired t-test (basal rate: 2.5 ± 0.6 spikes/s). Pre- *vs*. post-secretin during benoxathian microdialysis: P=0.1, paired t-test (basal rate: 1.7 ± 0.5 spikes/s). 0-5min after secretin during vehicle *vs.* benoxathian microdialysis: P=0.03, t-test. Benoxathian microdialysis significantly attenuated the secretin induced excitatory response in SON oxytocin neurones.

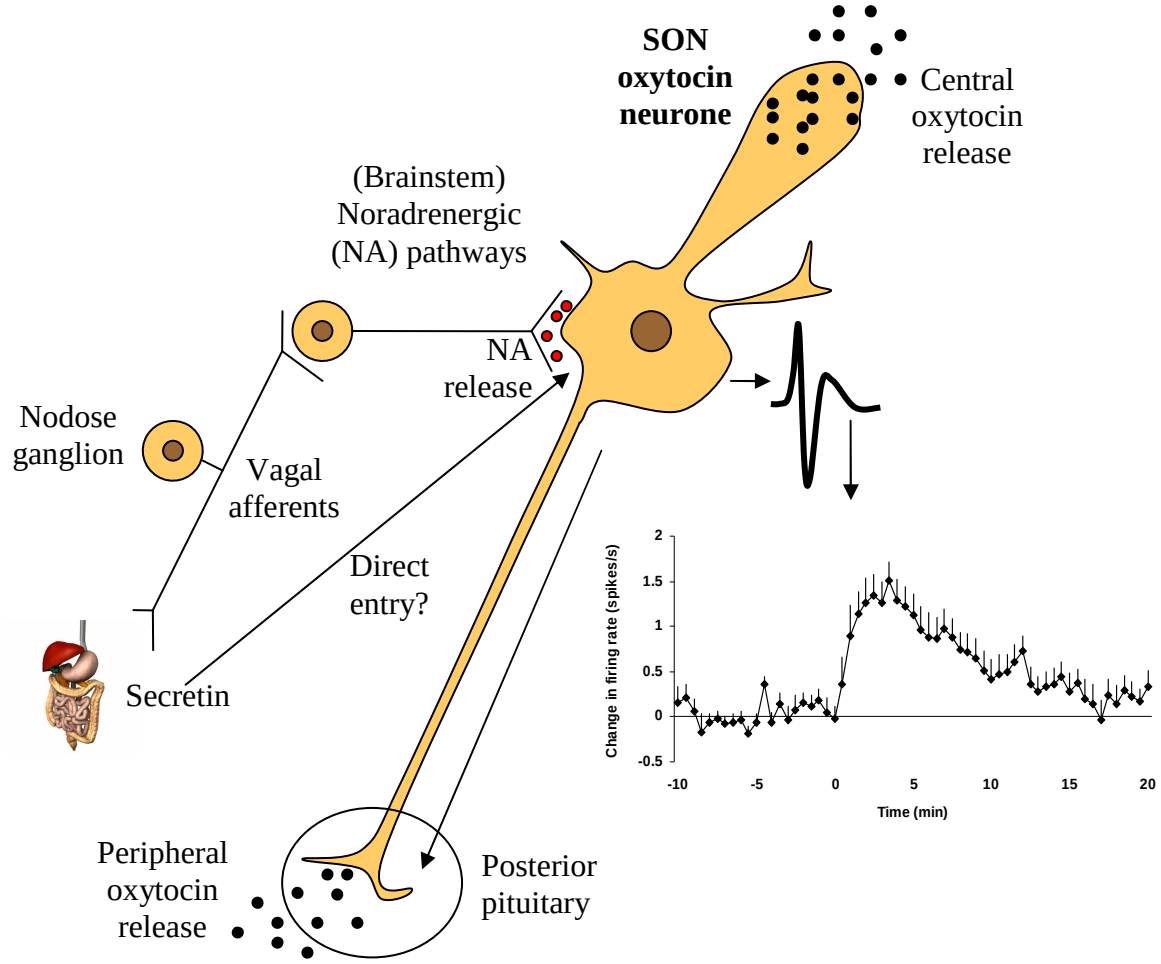


Fig. 5.35. Mode of action of systemic secretin on SON oxytocin neurones. Systemic secretin activates vagal afferents (Li et al., 2005) and, perhaps, subsequently the brainstem nuclei (Yang et al., 2004). SON receives noradrenergic inputs from the brainstem (Alonso and Assenmacher, 1984) that activates SON oxytocin neurones. The increase in electrical activity is accompanied by peripheral release of oxytocin into the circulation. Systemic secretin might also act directly on the secretin receptors on the SON neurones (Chu et al., 2006) bypassing blood-brain barrier (Banks et al., 2002).

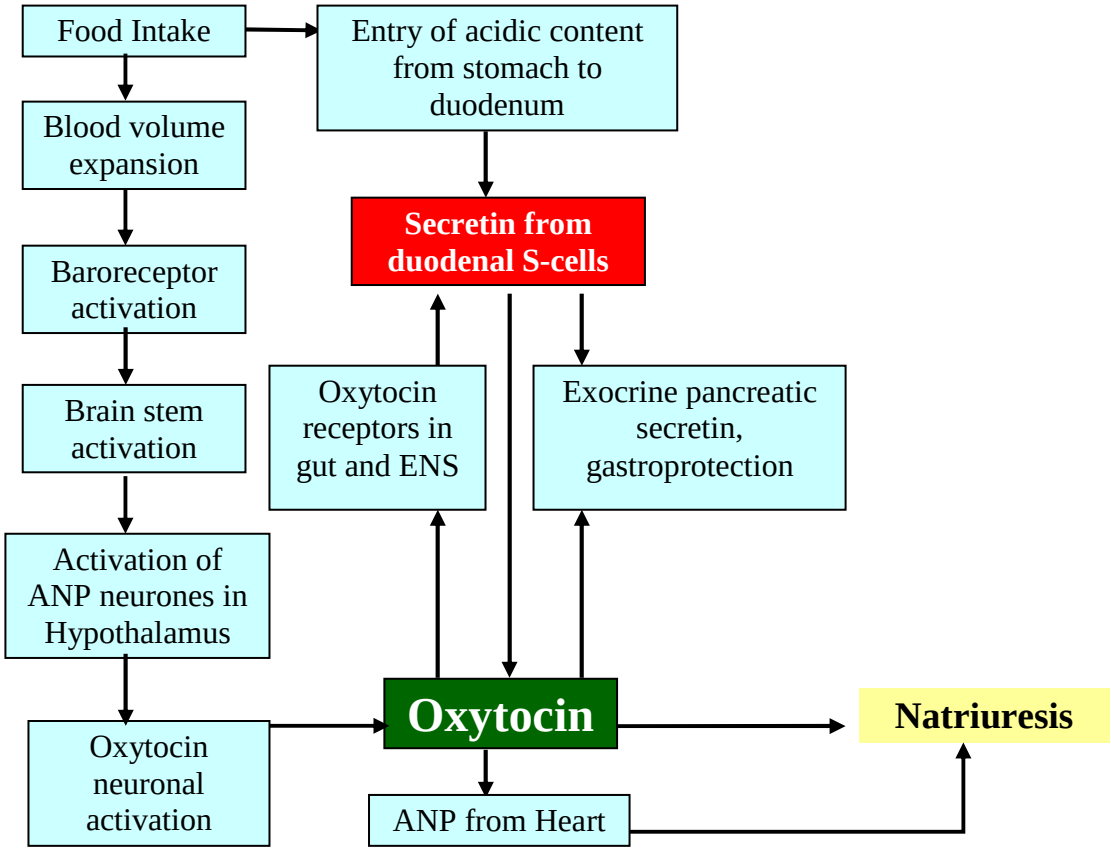


Fig. 5.36. Possible synergistic actions of secretin and oxytocin in gastrointestinal functions and water and electrolyte homeostasis. Upon entry of acidic contents from the stomach to the intestines, secretin is released from the duodenal S-cells (Oektedalen et al., 1982). Secretin increases exocrine pancreatic secretion containing water and bicarbonate to neutralise the acidity (Chey et al., 1984). Oxytocin is also released postprandially in response to blood volume expansion accompanying prandial drinking (Haanwinckel et al., 1995). A cascade of activation of brainstem and hypothalamic neurones ends up with peripheral oxytocin secretion that regulates natriuresis directly or indirectly (Haanwinckel et al., 1995). The results from the present study show the possibility that, physiologically, secretin might be another stimulus like CCK, in activating oxytocin neurones for postprandial regulation of fluid balance. Secretin itself is natriuretic (Waldum et al., 1980b; Waldum et al., 1980a; Waldum et al., 1981; Londong et al., 1987). Oxytocin, in turn, like secretin, protects the gastric mucosa (Asad et al., 2001) and might act on its receptors in the enteric nervous system (Welch et al., 2009) to regulate gastrointestinal functions.

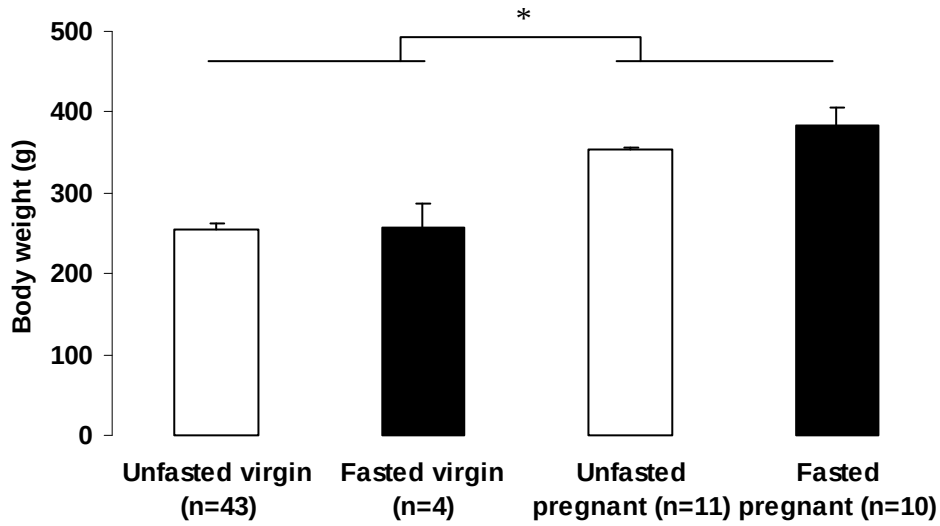


Fig. 6.1. Average body weight of the four groups of animals used in CCK study. Values are mean ± s.e.m. * P<0.001, $F_{1, 64}$=58.7, two-way ANOVA, virgin *vs.* pregnant groups. Pregnant rats were heavier than the virgin rats, whether they were fed or unfed.

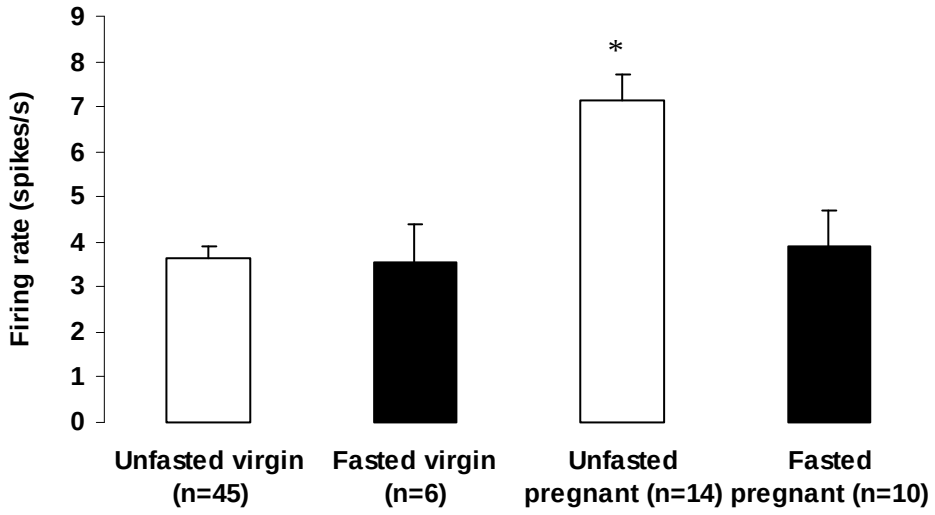


Fig. 6.2. Average basal firing rate of SON oxytocin neurones in the four groups of animals used in CCK study. Values are mean ± s.e.m. *P=0.001, $F_{1, 71}$=11.3, two-way ANOVA, pregnant group *vs.* other groups.

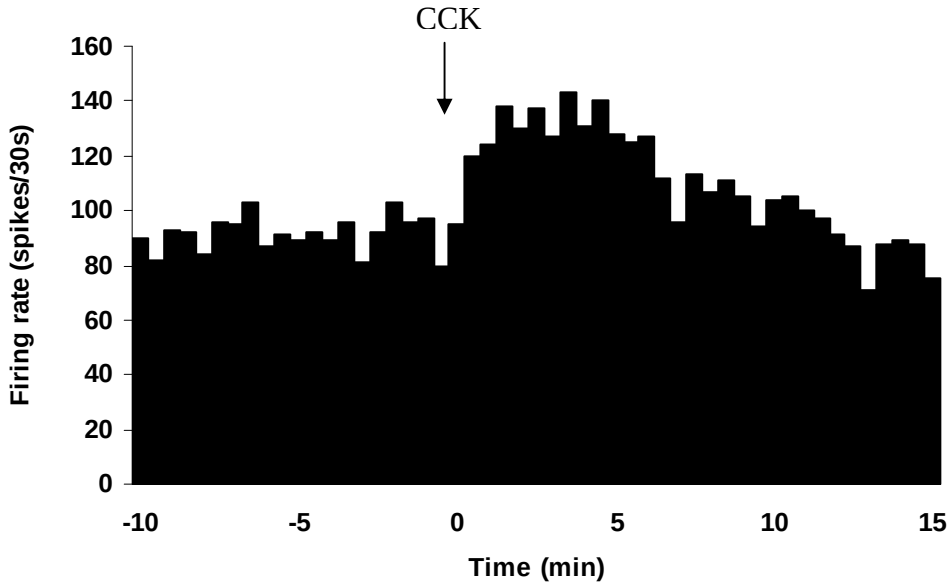


Fig. 6.3. The effect of CCK (25µg/kg; i.v) on the firing rate of a SON oxytocin neurone in an unfasted virgin rat (Cell No. 63-1). Basal firing rate of 3.05 spikes/s was increased by 1.6 spikes/s 1.5min after CCK. This increase in firing rate gradually returned to basal rate by ca. 12min.

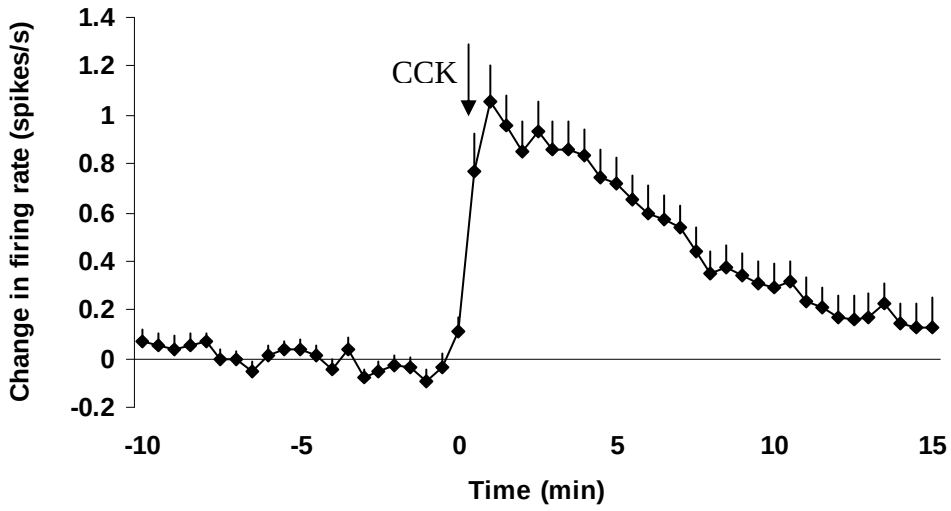


Fig. 6.4. The effect of systemic administration of CCK (25µg/kg; i.v) on the firing rate of SON oxytocin neurones in unfasted virgin rats (n=45). Values are mean ± s.e.m. Pre- *vs.* 0-10min post-CCK: $P<0.001$, Wilcoxon signed rank test. Basal firing rate: 3.6 ± 0.3 spikes/s. CCK administration significantly increased the firing rate of SON oxytocin neurones.

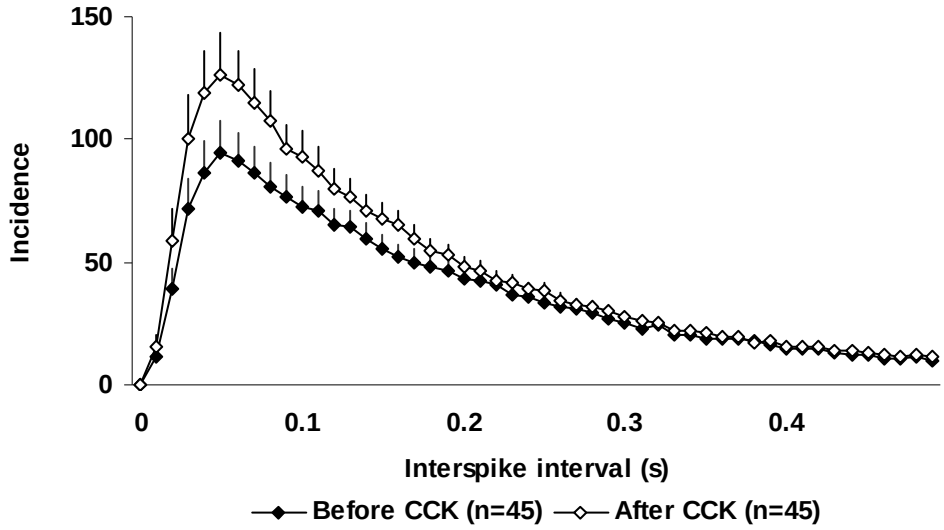


Fig. 6.5a. The effect of systemic administration of CCK (25µg/kg; i.v) on the interspike interval of SON oxytocin neurones in unfasted virgin rats. Values are mean ± s.e.m; not normalised. Incidence of interspike intervals occurring between 0–0.5s after a spike 0-10min after CCK *vs.* 10min basal period: P<0.001, Wilcoxon signed rank test. CCK administration significantly increased incidence of interspike intervals occurring between 0–0.5s after a spike.

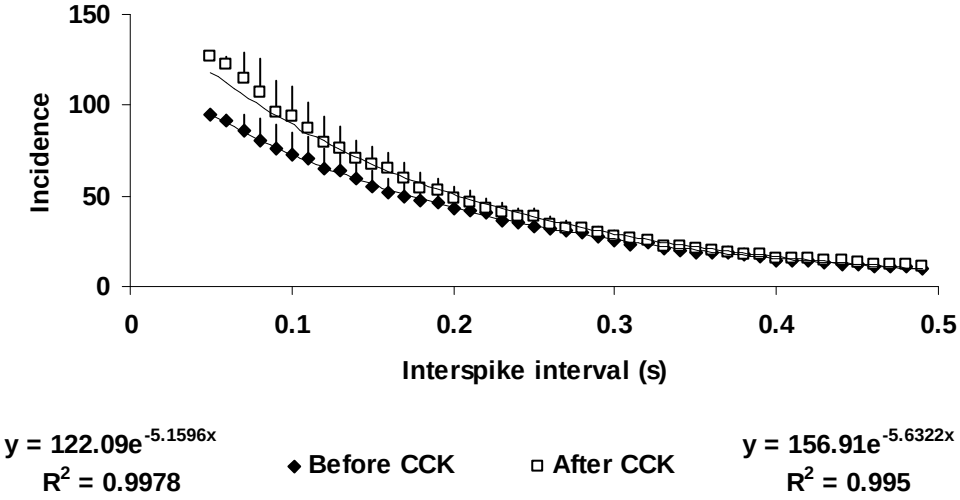


Fig. 6.5b. The interspike interval histograms before and after CCK fitted with exponential curves. The regression equations are $y = 122.09e^{-5.1596x}$ and $y = 156.91e^{-5.6322x}$. R^2 value of 0.99 indicates that the regression lines perfectly fit the data.

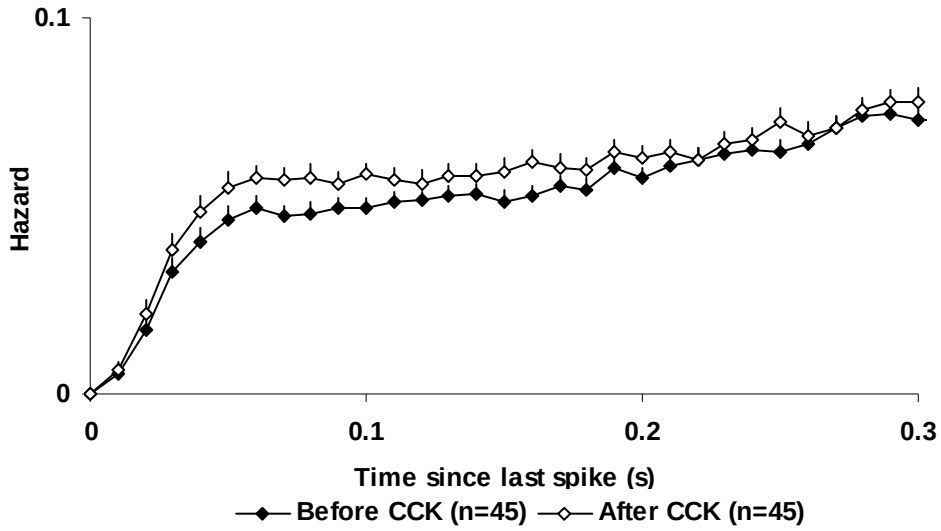


Fig. 6.6. The effect of CCK (25µg/kg; i.v) on the mean post-spike probability of SON oxytocin neurones in unfasted virgin rats (n=45): Hazard analysis. Values are mean ± s.e.m; not normalised. The comparison shows that the profile of activity-dependent changes in excitability is very similar before and after CCK, the main difference being a small change in the late hazard level. This suggests that the effects of CCK reflect an increase in synaptic input rather than a change in intrinsic voltage-dependent membrane properties.

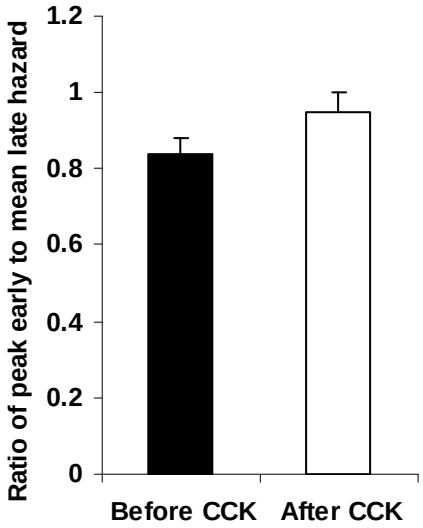


Fig. 6.7. The effect of CCK (25µg/kg; i.v) on the mean post-spike probability of SON oxytocin neurones in unfasted virgin rats (n=45): Ratio of peak early (<0.07s) to mean late (0.2-0.3s) hazard: Values are mean ± s.e.m. Pre- *vs.* 0-10min post-CCK: P=0.2, paired t-test. [n.s]

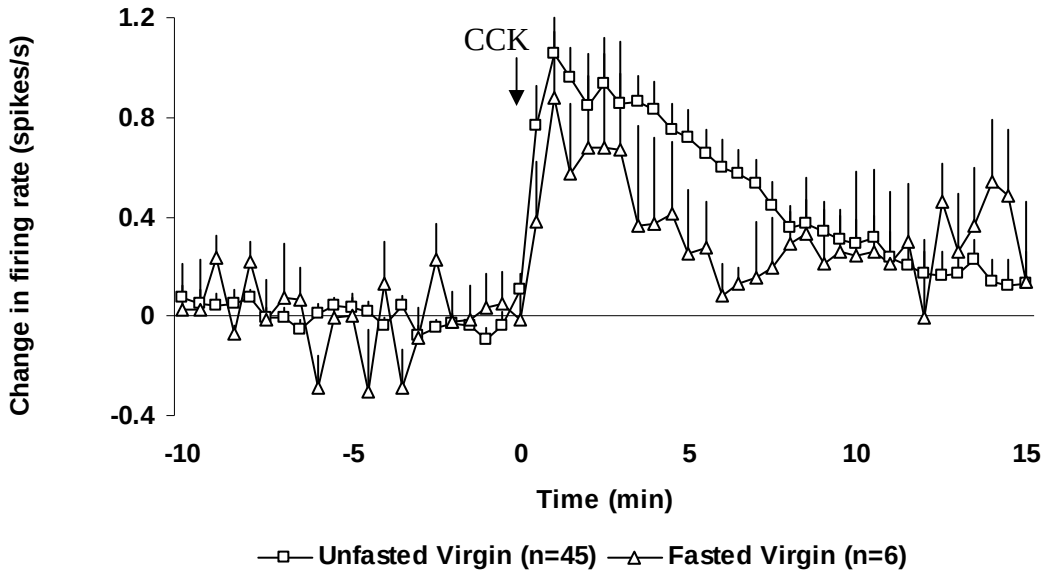


Fig. 6.8. The effect of CCK (25µg/kg; i.v) on the firing rate of SON oxytocin neurones in unfasted *vs.* fasted virgin rats. Values are mean ± s.e.m. Pre- *vs.* 0-10min post-CCK within the unfasted virgin group: P<0.001, Wilcoxon signed rank test. Pre- *vs.* 0-10min post-CCK within the fasted virgin group: P=0.06, paired t-test. 0-10min after CCK between the unfasted and fasted virgin groups: P=0.4; t-test. Basal firing rates (unfasted virgin: 3.6 ± 0.3 spikes/s; fasted virgin: 3.5 ± 0.9 spikes/s) did not differ significantly (P=0.9, t-test) between the groups.

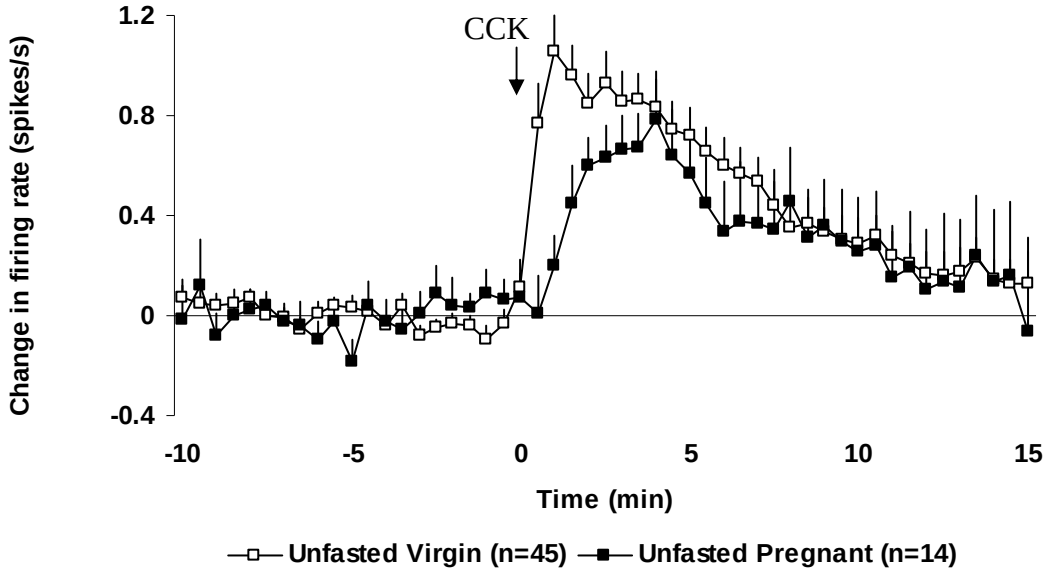


Fig. 6.9. The effect of CCK (25µg/kg; i.v) on the firing rate of SON oxytocin neurones in unfasted virgin *vs.* pregnant rats. Values are mean ± s.e.m. Pre- vs 0-10min post-CCK within the unfasted virgin group: P<0.001, Wilcoxon signed rank test. Pre- vs 0-10min post-CCK within the unfasted pregnant group: P=0.005, paired t-test. 0-2min after CCK between unfasted virgin and pregnant groups: P=0.012, t-test (0-10min after CCK: P=0.2, t-test). Basal firing rates (unfasted virgin: 3.6 ± 0.3 spikes/s; unfasted pregnant: 7.2 ± 0.6 spikes/s) differed significantly (P<0.001, t-test) between the groups.

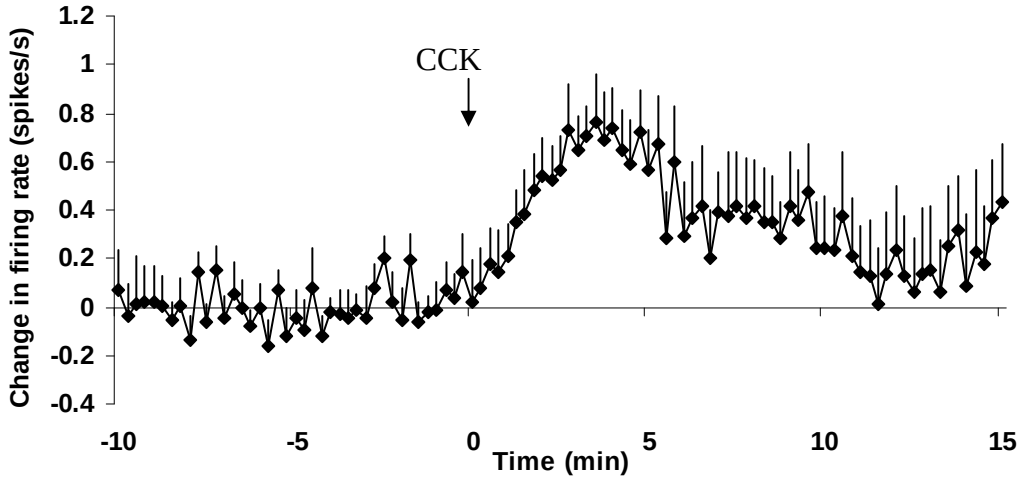


Fig. 6.9a. The effect of CCK (25µg/kg; i.v) on the firing rate of SON oxytocin neurones in unfasted pregnant rats: 15s bin. The response to CCK begins immediately after CCK; however, the time to reach the peak excitation is gradual and delayed, unlike in virgin rats in which the peak excitation was reached rapidly within 2min.

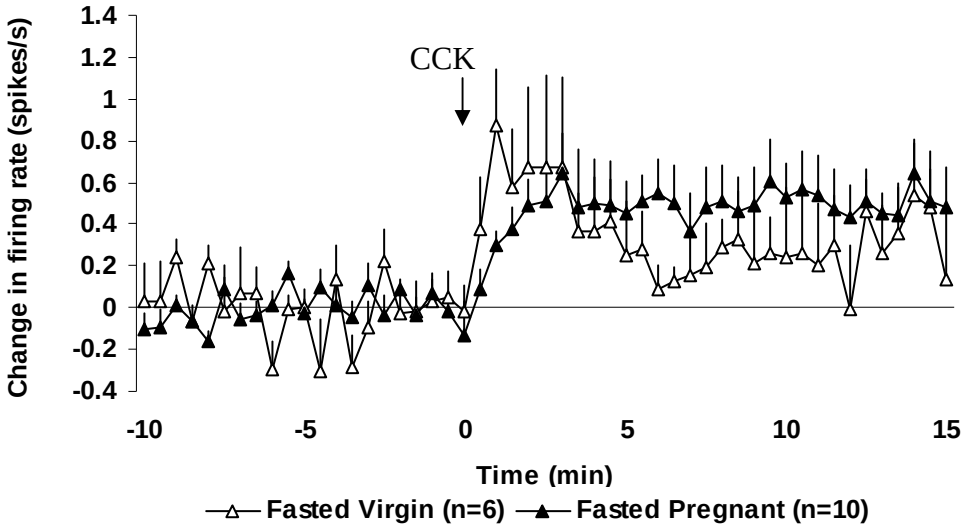


Fig. 6.10. The effect of CCK (25µg/kg; i.v) on the firing rate of SON oxytocin neurones in fasted virgin *vs.* pregnant rats. Values are mean ± s.e.m. Pre- *vs.* 0-10min post-CCK within the fasted pregnant group: P=0.002, paired t-test. Pre- *vs.* 0-10min post-CCK within the fasted virgin group: P=0.06, paired t-test. 0-10min after CCK between the fasted virgin and pregnant groups: P=0.99, t-test. Basal firing rates (fasted virgin: 3.5 ± 0.9 spikes/s; fasted pregnant: 3.9 ± 0.8 spikes/s) did not differ significantly (P=0.8, t-test) between the groups.

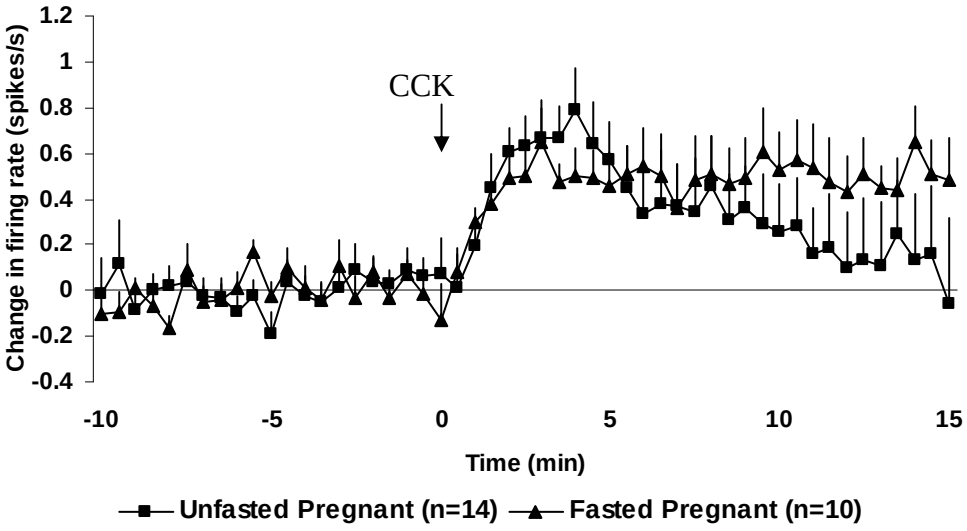


Fig. 6.11. The effect of CCK (25µg/kg; i.v) on the firing rate of SON oxytocin neurones in unfasted and fasted pregnant rats. Values are mean ± s.e.m. Pre- *vs*. 0-10min post-CCK within the unfasted pregnant group: P=0.005, paired t-test. Pre- *vs.* 0-10min post-CCK within the fasted pregnant group: P=0.002, paired t-test. 0-10min after CCK between unfasted and fasted pregnant groups: P=0.9, t-test. Basal firing rates (unfasted pregnant: 7.2 ± 0.6 spikes/s; fasted pregnant: 3.9 ± 0.8 spikes/s) differed significantly (p=0.002, t-test) between the groups.

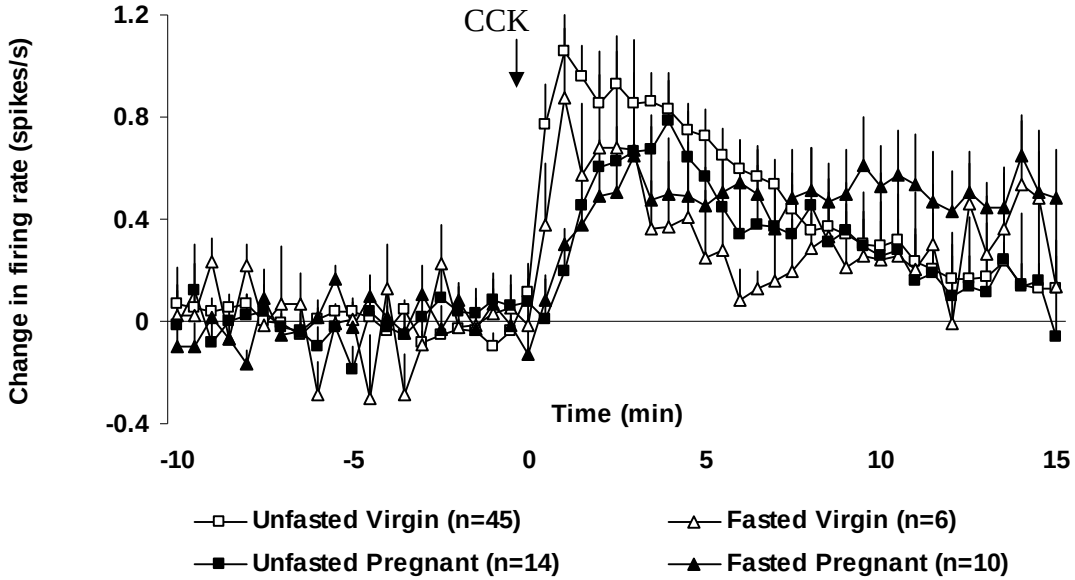


Fig. 6.12. The effect of CCK (25µg/kg; i.v) on the firing rate of SON oxytocin neurones in unfasted/fasted virgin/pregnant rats. Values are mean ± s.e.m. P=<0.001, Wilcoxon signed rank test, pre- *vs.* 0-10min post-CCK within the unfasted virgin group. P=0.005, paired t-test, pre- *vs.* 0-10min post-CCK within the unfasted pregnant group. P=0.002, paired t-test, pre- *vs.* 0-10min post-CCK within the fasted pregnant group. Pre- *vs.* 0-10min post-CCK within the fasted virgin group: P=0.06, paired t-test. Basal firing rate of unfasted pregnant group (7.2 ± 0.6 spikes/s) differed significantly from all other groups (unfasted virgin: 3.6 ± 0.3 spikes/s; fasted virgin: 3.5 ± 0.9 spikes/s; fasted pregnant: 3.9 ± 0.8 spikes/s; P=0.001, $F_{1, 71}$=11.3, two-way ANOVA). The CCK-induced excitatory responses observed in unfasted/fasted virgin/pregnant rats during 0-10min after CCK injection were not different from each other Hence, there was no interaction between feeding and reproductive status in the CCK-induced responses ($F_{1, 65}$=0.01, P=0.9, two-way ANOVA).

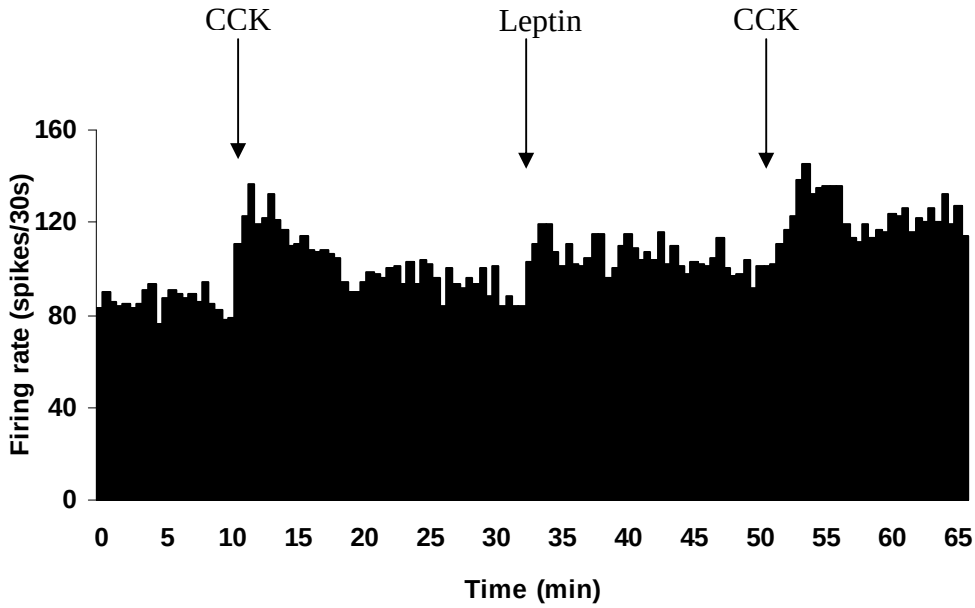


Fig. 6.13. Effect of leptin administration (100µg; i.v) on the CCK-induced (25µg/kg; i.v) response of a SON oxytocin neurone in a virgin female rat (No. 235-1).

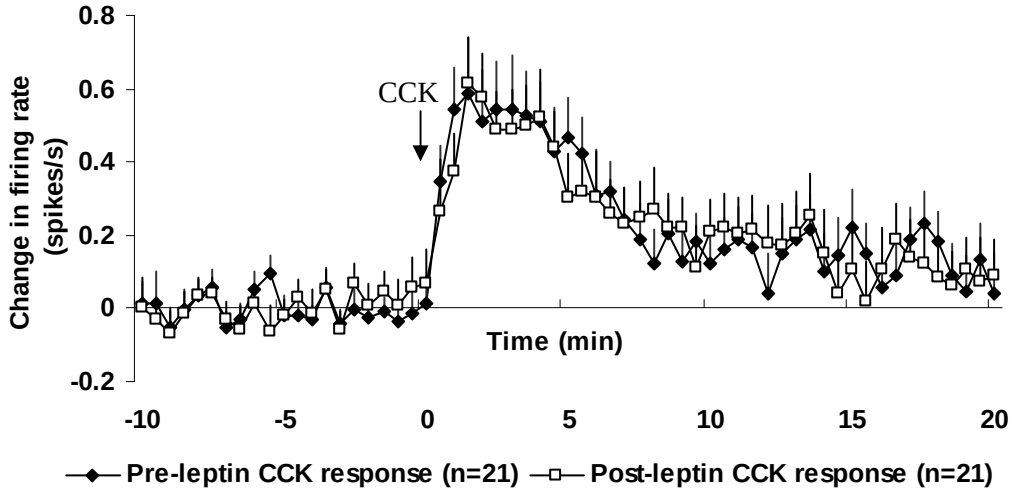


Fig. 6.14. Effect of leptin (100µg; i.v) on CCK-induced (25µg/kg; i.v) response of SON oxytocin neurones. Values are mean ± s.e.m. $P<0.001$, paired t-test, 0-10min responses after CCK *vs.* respective basal. Basal rates (2.9 ± 0.3 spikes/s and 3 ± 0.4 spikes/s) did not differ from each other ($P=0.9$, paired t-test). The CCK-induced excitatory responses before and after leptin were not different from each other ($P=0.8$, t-test).

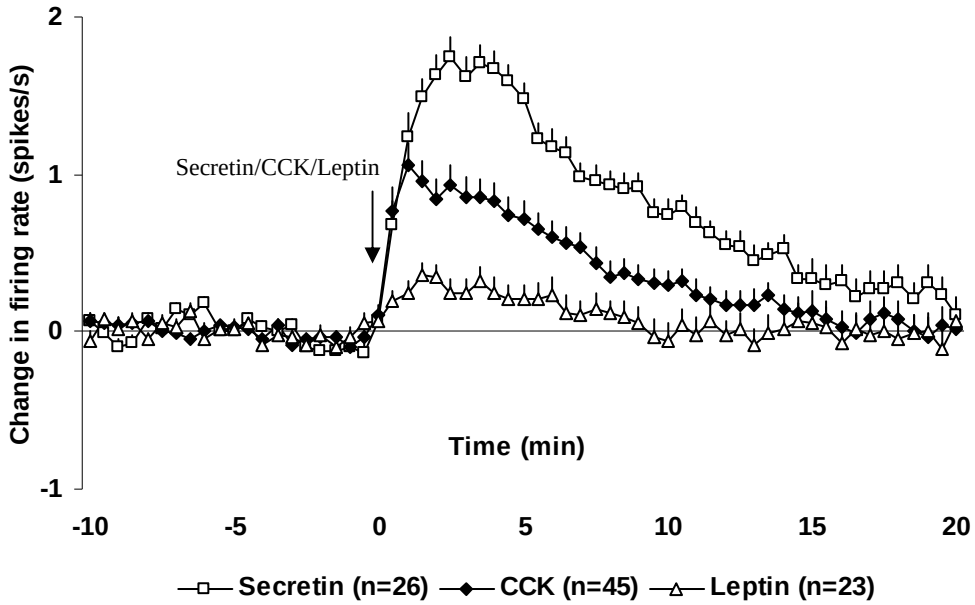


Fig. 6.15. Effect of secretin (0.1µg/rat; i.v), leptin (100µg/rat; i.v) and CCK (25µg/kg; i.v) on the electrical activity of SON oxytocin neurones in unfasted virgin rats. Values are mean ± s.e.m. P<0.001, paired t-test, pre- *vs.* 0-10min post-secretin within the secretin group. P<0.001; Wilcoxon signed rank test, pre- *vs.* 0-10min post-CCK within the CCK group. P=0.01; paired t-test, pre- *vs.* 0-10min post-leptin within the leptin group. P<0.001, Kruskal-Wallis one-way ANOVA on ranks, 0-10min after administration all peptides between the groups. Basal firing rates (secretin group: 4.1 ± 0.4 spikes/s; leptin group: 3.4 ± 0.4 spikes/s; CCK group: 3.6 ± 0.3 spikes/s) did not differ significantly (P=0.3, one-way ANOVA). In comparison with CCK, secretin was the most effective and leptin was the least effective peptide in terms of excitation of SON oxytocin neurones.

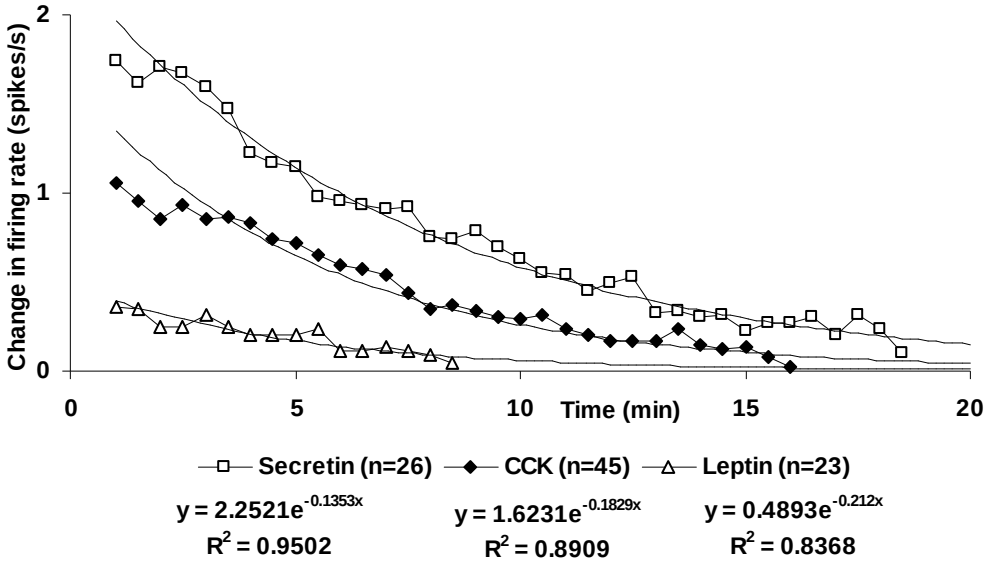


Fig. 6.16. The change in firing rate after secretin/CCK/leptin administration fitted with exponential curves. The half-lives of the electrical activity calculated from this for these three peptides are: secretin: 5.1min, CCK: 3.8min, and leptin: 3.3min.

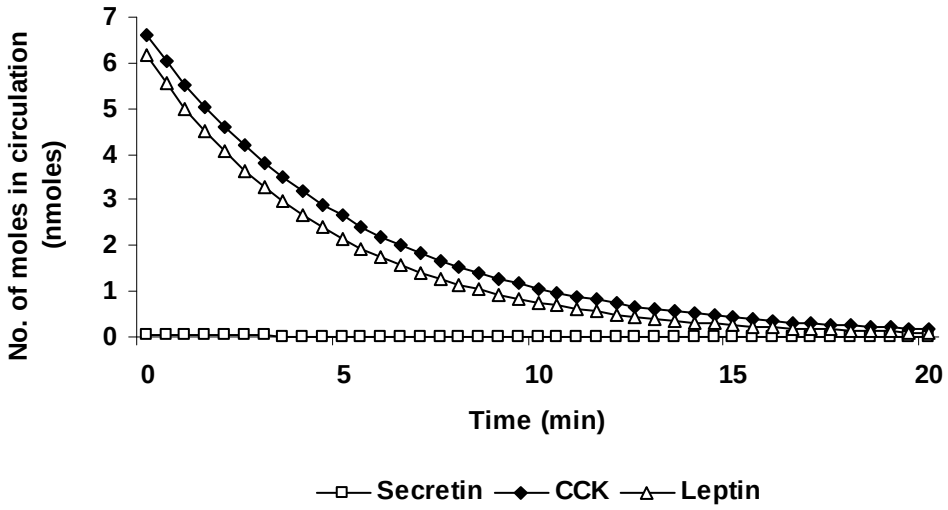


Fig. 6.17a. The decay of secretin, CCK and leptin in the circulation. The number of moles of secretin, CCK and leptin administered initially are 0.033, 6.6 and 6.17 nmoles, respectively. The decay of these peptides in the circulation was calculated from the half-lives of the firing rate. The decay for each peptide is given in the following figures.

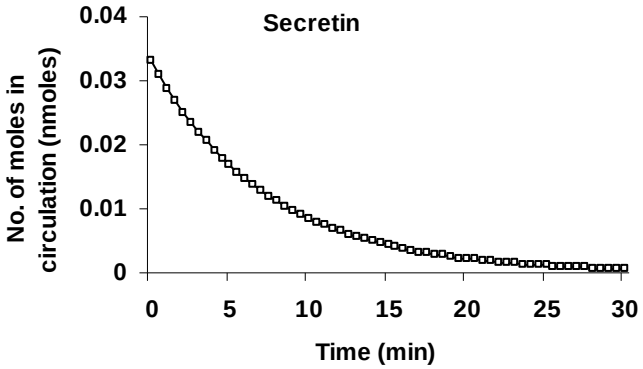


Fig. 6.17b. The decay of secretin in the circulation. The decay of circulating secretin was calculated from the decrease in firing rate after peak excitation. Firing rate reaches basal by 20min while the secretin level in the circulation decayed to near 0 only by 30min.

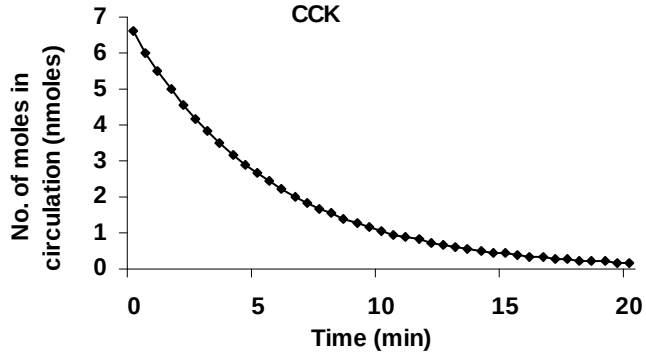


Fig. 6.17c. The decay of CCK in the circulation. The decay of circulating CCK was calculated from the decrease in firing rate after peak excitation. Firing rate reaches basal by 16min while the CCK level in the circulation decayed to near 0 only by 20min.

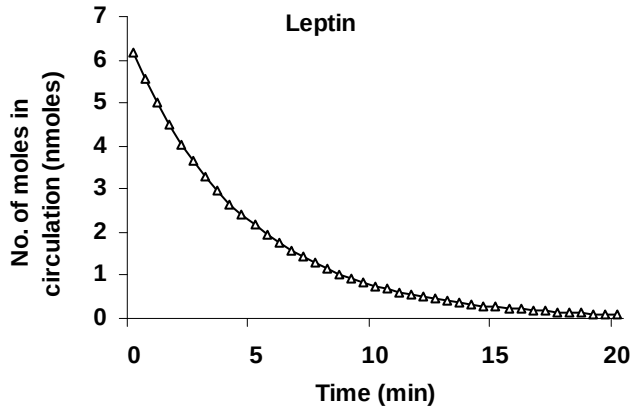


Fig. 6.17d. The decay of leptin in the circulation. The decay of circulating leptin was calculated from the decrease in firing rate after peak excitation. Firing rate reaches basal by 10min while the leptin level in the circulation decayed to near 0 only by 18min.

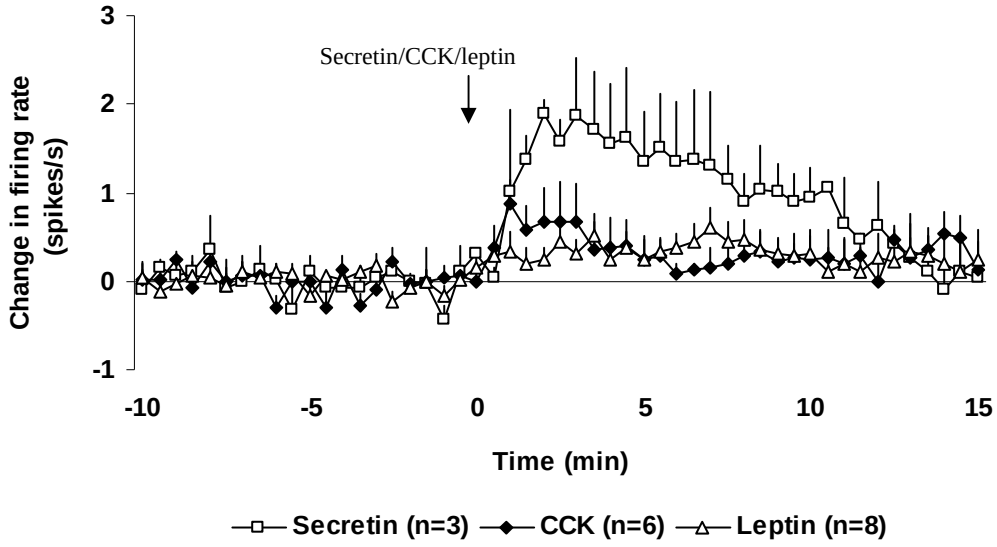


Fig. 6.18. Effects of secretin (0.1µg/rat; i.v), leptin (100µg/rat; i.v) and CCK (25µg/kg; i.v) on the electrical activity of SON oxytocin neurones in fasted virgin rats. Values are mean ± s.e.m. P=0.047; paired t-test, pre- *vs.* 0-10min post-secretin within the secretin group. Pre *vs.* 0-10min post-leptin within the leptin group: P=0.05, paired t-test. Pre- *vs.* 0-10min post-CCK within the CCK group: P=0.06, paired t-test. 0-10min after the administration of peptides between the groups: P=0.03, one-way ANOVA (Holm-Sidak multiple comparison test: secretin *vs.* CCK: P=0.01; secretin *vs.* leptin: P=0.02). Between fasted and unfasted groups: Secretin response: 0.9, t-test; CCK response: P=0.4, Mann-Whitney rank sum test; leptin response: P=0.2, t-test. Basal firing rates (secretin group: 3.8 ± 0.6 spikes/s; leptin group: 4.5 ± 0.8 spikes/s; CCK group: 3.5 ± 0.9 spikes/s) did not differ between fasted virgin groups (P=0.8, one-way ANOVA) and did not differ from the unfasted virgin groups (P=0.7, one-way ANOVA). The excitatory responses of SON oxytocin neurones to secretin, leptin and CCK in fasted rats were not different from unfasted rats.

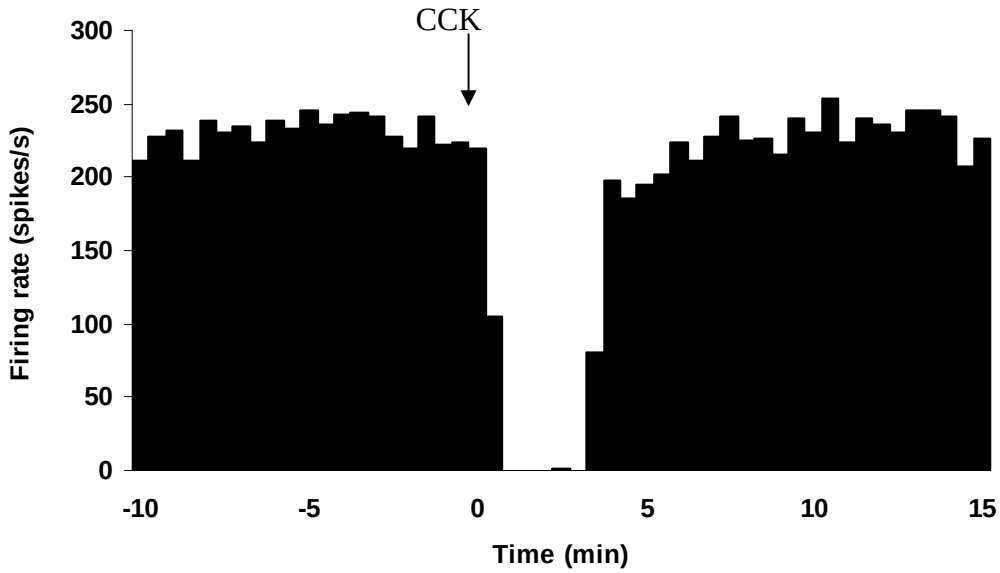


Fig. 6.19. The effect of CCK (25µg/kg; i.v) on the firing rate of a non-phasic SON vasopressin neurone in an unfasted virgin rat (Cell No. 15-4). The basal firing rate was 7.7 spikes/s. After CCK administration, the cell remained inhibited for ca. 3min after which it resumed its original firing rate.

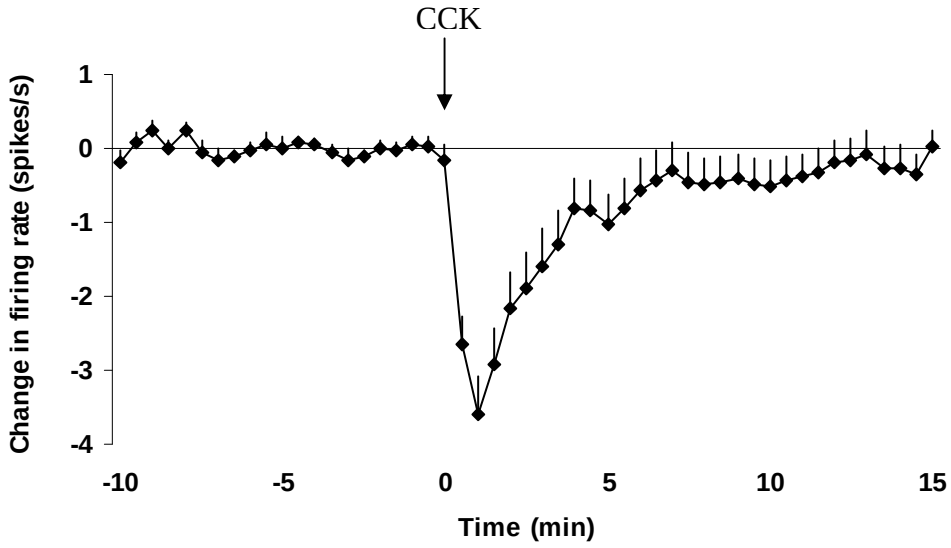


Fig. 6.20. The effect of CCK (25µg/kg; i.v) on the firing rate of non-phasic SON vasopressin neurones in unfasted virgin rats (n=23). Values are mean ± s.e.m. Pre- *vs.* 0-5min post-CCK: $P<0.001$, paired t-test. The basal firing rate of 6.1 ± 0.5 spikes/s was decreased by 3.6 ± 0.5 spikes/s 1min after CCK. The rate gradually returned to basal rate by 15min. These twenty three of 30 non-phasic vasopressin neurones were inhibited by CCK while the seven other neurones did not respond to CCK.

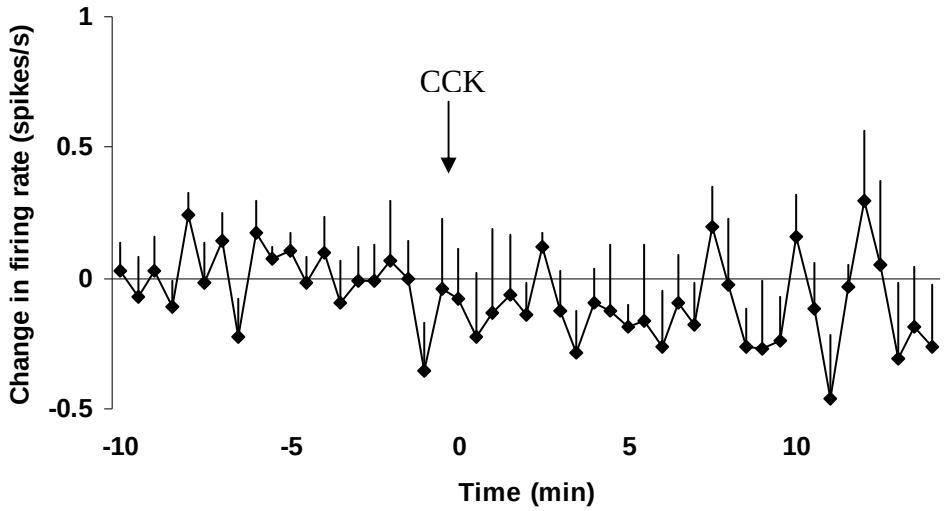


Fig. 6.21. The effect of CCK (25µg/kg; i.v) on the unresponsive non-phasic SON vasopressin neurones in unfasted virgin rats (n=7). Values are mean ± s.e.m. In these neurones, the basal firing rate (4.6 ± 1.4 spikes/s) was not altered by CCK. [n.s]

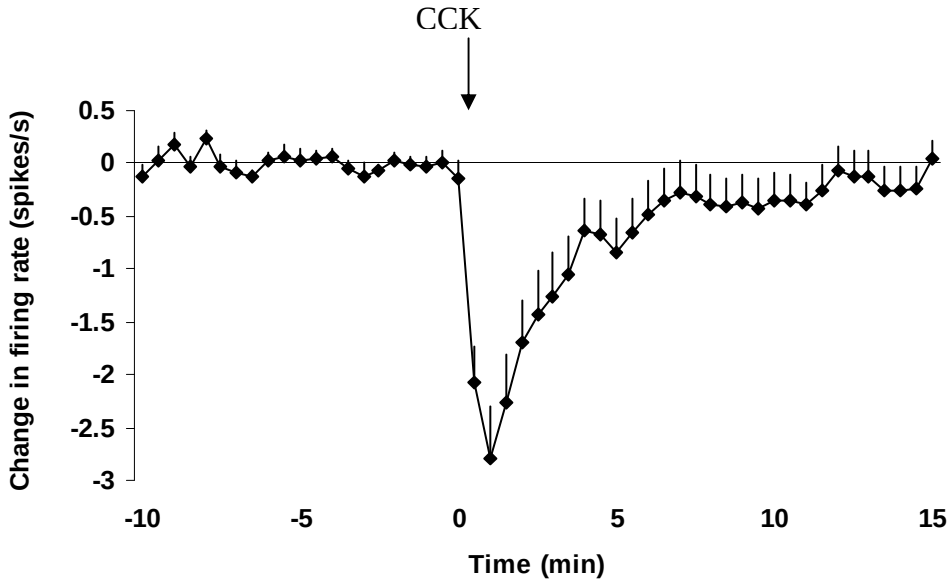


Fig. 6.22. The effect of systemic administration of CCK (25µg/kg; i.v) on the firing rate of all recorded non-phasic SON vasopressin neurones in unfasted virgin rats (n=30): Values are mean ± s.e.m. Basal firing rate: 5.7 ± 0.5 spikes/s. Pre- *vs.* 0-5min post-CCK: $P<0.001$, Wilcoxon signed rank test. Twenty three of 30 non-phasic vasopressin neurones were inhibited by CCK while the seven other neurones did not respond to CCK. On average, CCK significantly inhibited non-phasic SON vasopressin neurones.

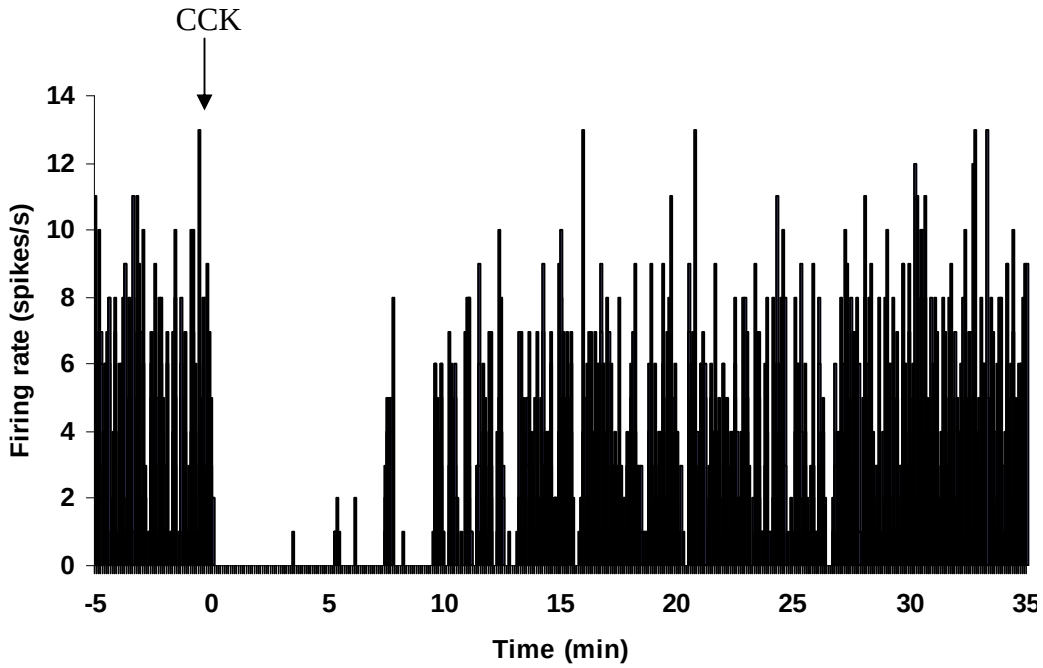


Fig. 6.23. The effect of CCK (25µg/kg; i.v) on phasic SON vasopressin neurone in an unfasted virgin rat (Cell No. 9-5). The activity quotient, frequency within bursts and mean interburst interval during the basal period were 0.03, 4.3 spikes/s and 10.8s, respectively. CCK completely arrested the activity of this cell during 0-5min after administration.

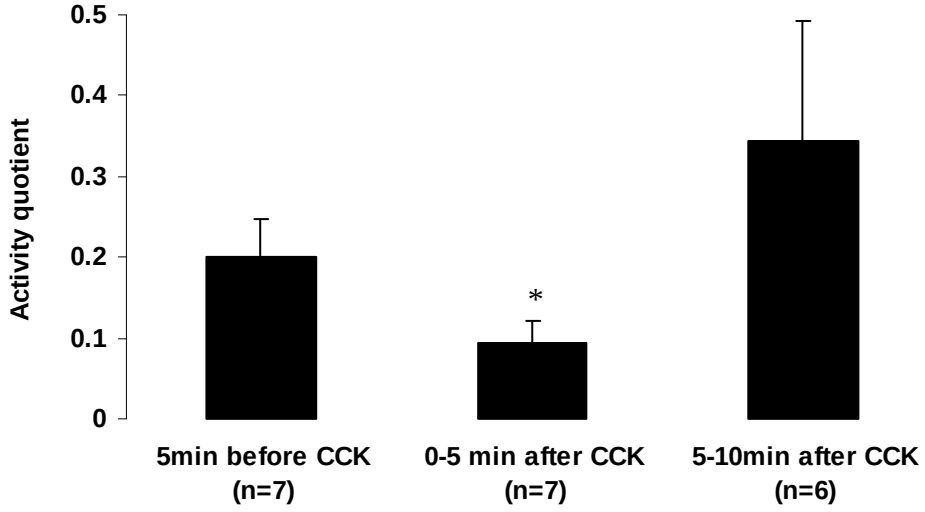


Fig. 6.24. The effect of CCK (25µg/kg; i.v) on phasic SON vasopressin neurones in unfasted virgin rats: Activity quotient. Values are mean ± s.e.m. * P=0.02, paired t-test, pre- *vs*. 0-5min post-CCK. CCK significantly decreased the mean activity quotient of phasic SON vasopressin neurones.

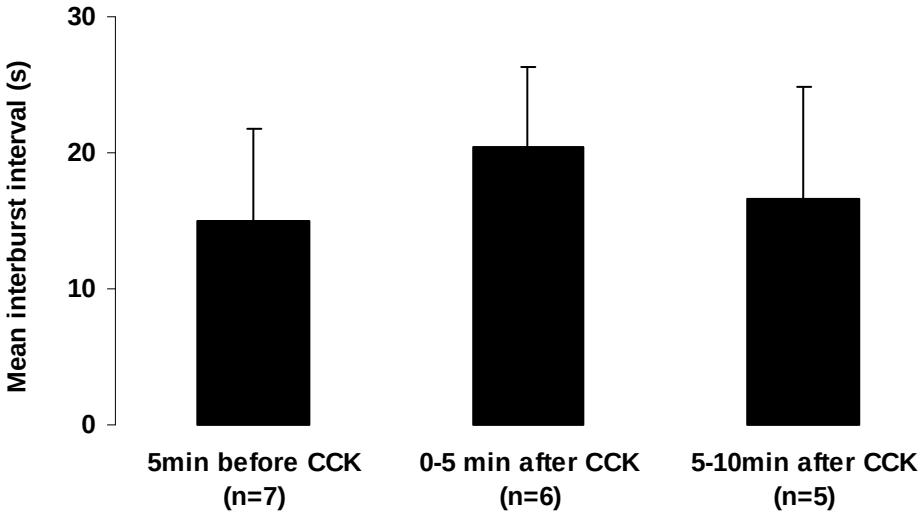


Fig. 6.25. Effect of CCK (25µg/kg; i.v) on phasic SON vasopressin neurones in unfasted virgin rats: Mean interburst interval (s). Values are mean ± s.e.m. Pre- *vs*. 0-5min post-CCK: P=0.3, paired t-test. There was no change in the mean interburst interval after CCK administration. [n.s]

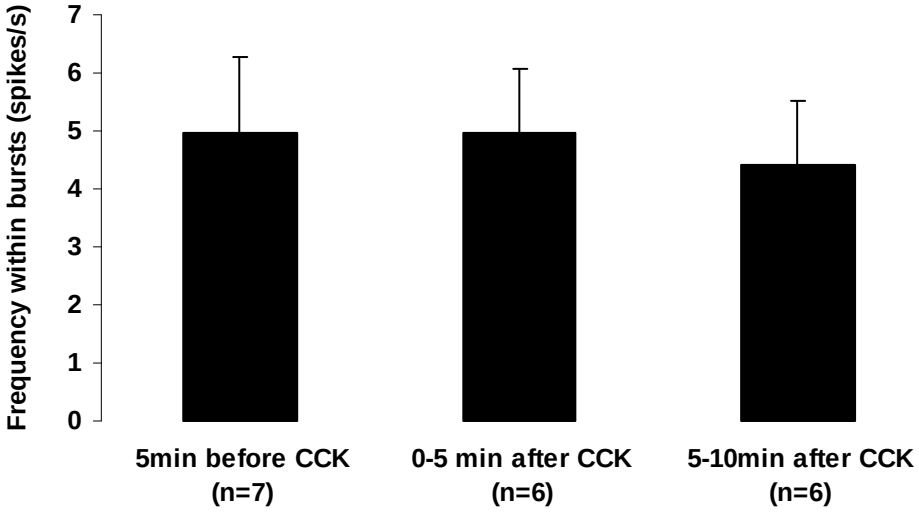


Fig. 6.26. The effect of CCK (25µg/kg; i.v) on phasic SON vasopressin neurones in unfasted virgin rats: Frequency within bursts (spikes/s). Values are mean ± s.e.m. Pre- *vs.* 0-5min post-CCK: P=0.8, paired t-test. There was no change in the frequency within bursts after CCK administration. [n.s]

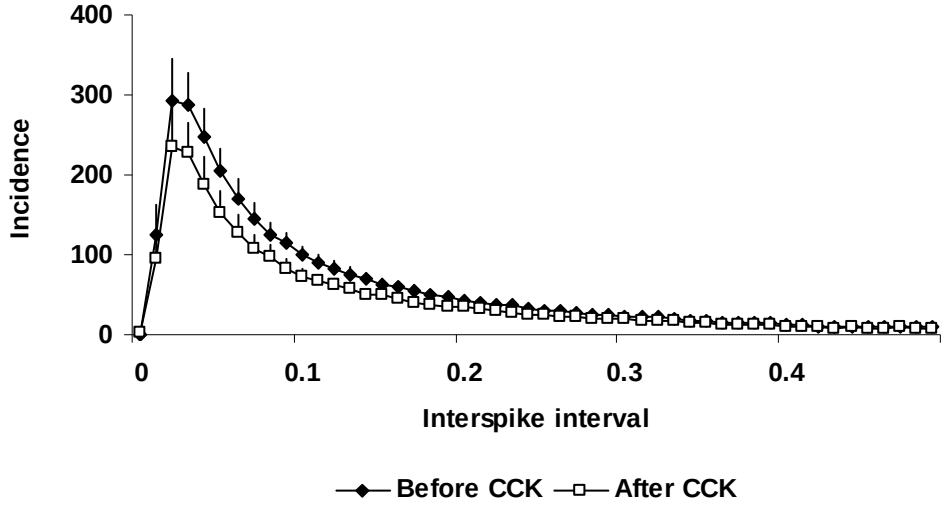


Fig. 6.27a. Effect of systemic administration of CCK on the interspike interval of SON vasopressin neurones (n=37; 30 non-phasic and 7 phasic vasopressin neurones). Values are mean ± s.e.m; not normalised. The number of spikes occurring with the interspike interval of less than 0.3s is reduced following CCK administration.

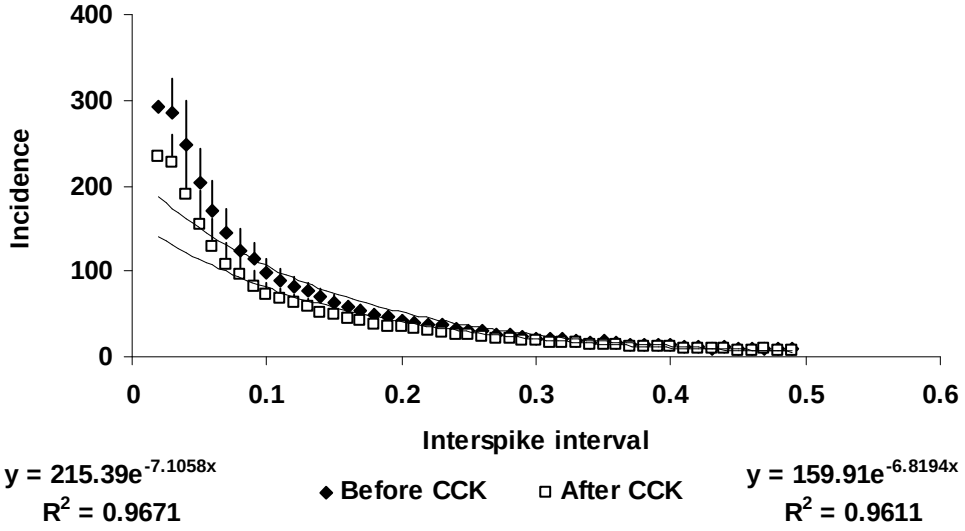


Fig. 6.27b. The interspike interval histograms before and after CCK fitted with exponential curves. The distal tail of the histograms fitted well with exponential curves while the intervals of less than 0.2s were left unfit, which is a characteristic of vasopressin neurones (n=37; 30 non-phasic and 7 phasic vasopressin neurones).

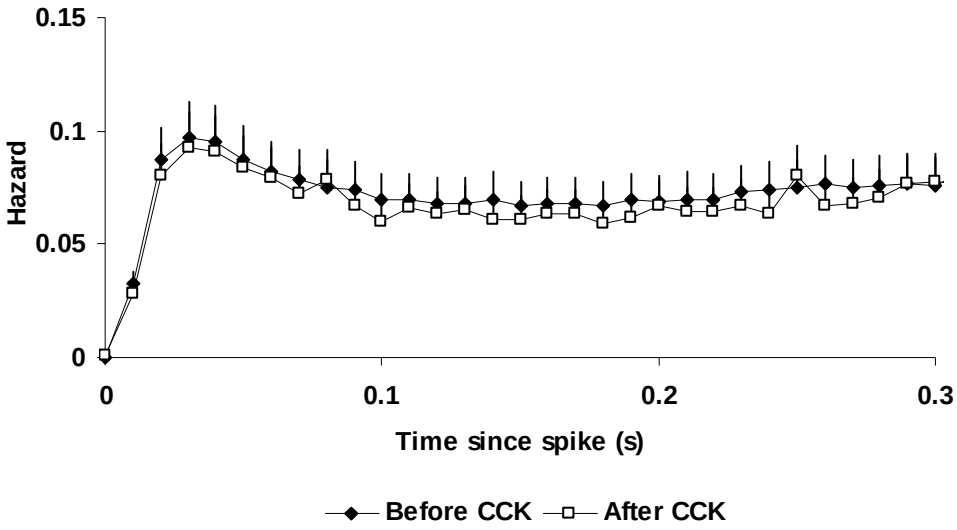


Fig. 6.28. Effect of systemic administration of CCK on the mean post-spike probability of SON vasopressin neurones (n=37; 30 non-phasic and 7 phasic vasopressin neurones). Values are mean ± s.e.m; not normalised. There was no change in the shape of the hazard plot following CCK.

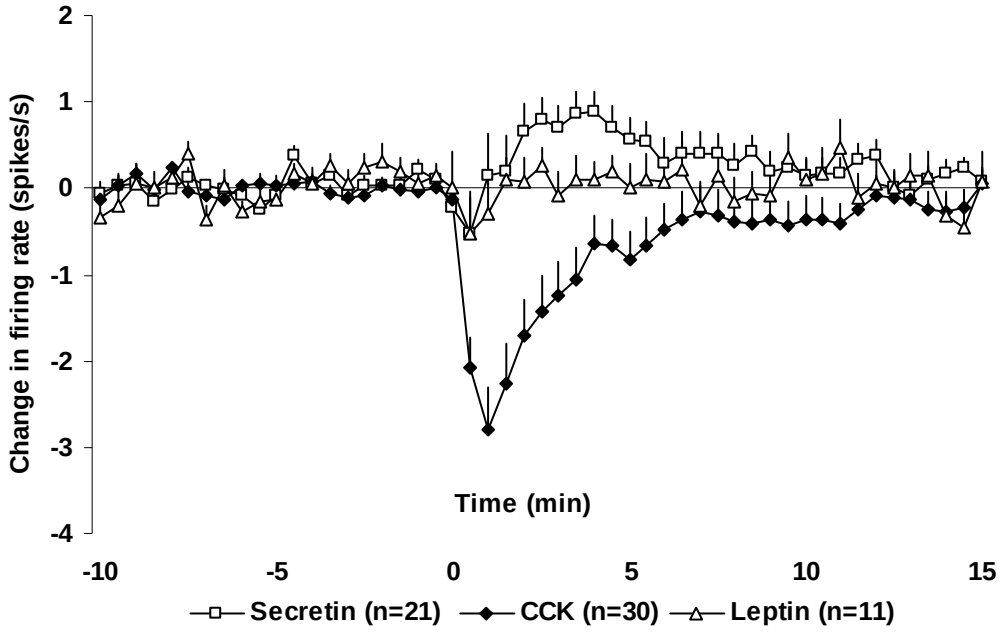


Fig. 6.29. Effect of secretin (0.1µg/rat; i.v), leptin (100µg/rat; i.v) and CCK (25µg/kg; i.v; n=30) on the electrical activity of non-phasic SON vasopressin neurones in unfasted virgin rats. Values are mean ± s.e.m. P<0.001, Wilcoxon signed rank test, pre- *vs.* 0-5min post-CCK within the CCK group. Pre- *vs.* 0-5min post-secretin within the secretin group: P=0.1, paired t-test. Pre- *vs.* 0-5min post-leptin within the leptin group: P=0.9, paired t-test. 0-5min after administration of peptides between the groups: P<0.001, Kruskal-Wallis one-way ANOVA on ranks (multiple comparison procedures by Dunn's Method: CCK *vs.* secretin and CCK *vs.* leptin: p<0.05). The basal firing rates (secretin group: 6.3 ± 0.6 spikes/s; CCK group: 5.7 ± 0.5 spikes/s and leptin group: 8.8 ± 1.4 spikes/s) did not differ between groups (P=0.1, Kruskal-Wallis one-way ANOVA on ranks). CCK significantly inhibited while secretin and leptin did not have much effect on the non-phasic vasopressin neurones.

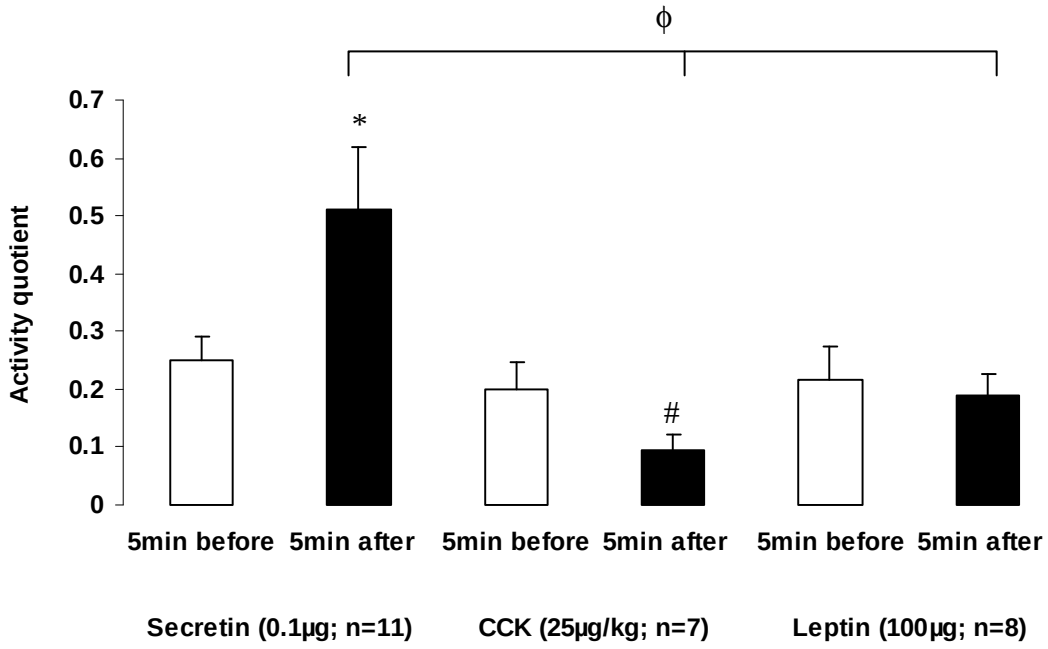


Fig. 6.30. Effects of secretin, leptin and CCK on phasic SON vasopressin neurones in unfasted virgin rats: Activity quotient. Values are mean ± s.e.m. * P=0.02, paired t-test, pre- *vs*. 0-5min post-secretin within the secretin group. # P=0.02, paired t-test, pre- *vs*. 0-5min post-CCK within the CCK group. Pre- *vs*. 0-5min post-leptin: P=0.3, paired t-test. ϕ P=0.03, Kruskal-Wallis one-way ANOVA on ranks (multiple comparison procedures by Dunn's Method: CCK *vs*. secretin: P<0.05), activity quotient 0-5min after administration of peptides between the groups. Basal activity quotient did not differ between the groups: P=0.8, Kruskal-Wallis one-way ANOVA on ranks. Secretin significantly increased and CCK significantly decreased the activity quotient of phasic SON vasopressin neurones while leptin did not have any effect.

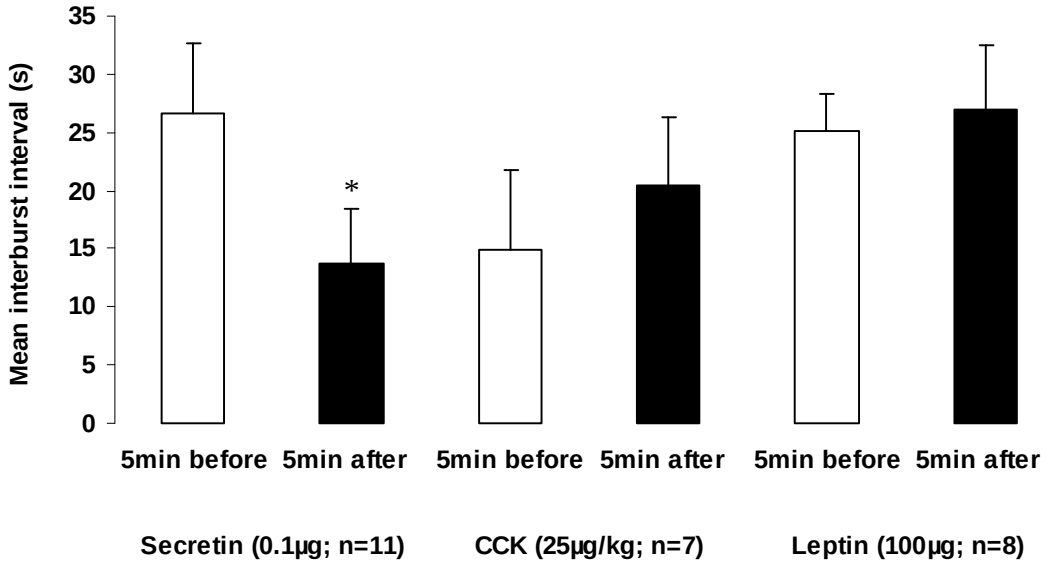


Fig. 6.31. Effect of secretin, leptin and CCK on phasic SON vasopressin neurones in unfasted virgin rats: Mean interburst interval (s). Values are mean ± s.e.m. * P=0.01, Wilcoxon signed rank test, pre- *vs.* 0-5min post-secretin within the secretin group. Pre- *vs.* 0-5min post-CCK within the CCK group: P=0.4, paired t-test. Pre- *vs*. 0-5min post-leptin within the leptin group: P=0.7, Wilcoxon signed rank test. Mean interburst interval during the basal period and 0-5min after administration of peptides did not differ between the groups: P=0.25 and P=0.2, one-way ANOVA. Secretin significantly decreased the mean interburst interval while leptin and CCK did not have any effect.

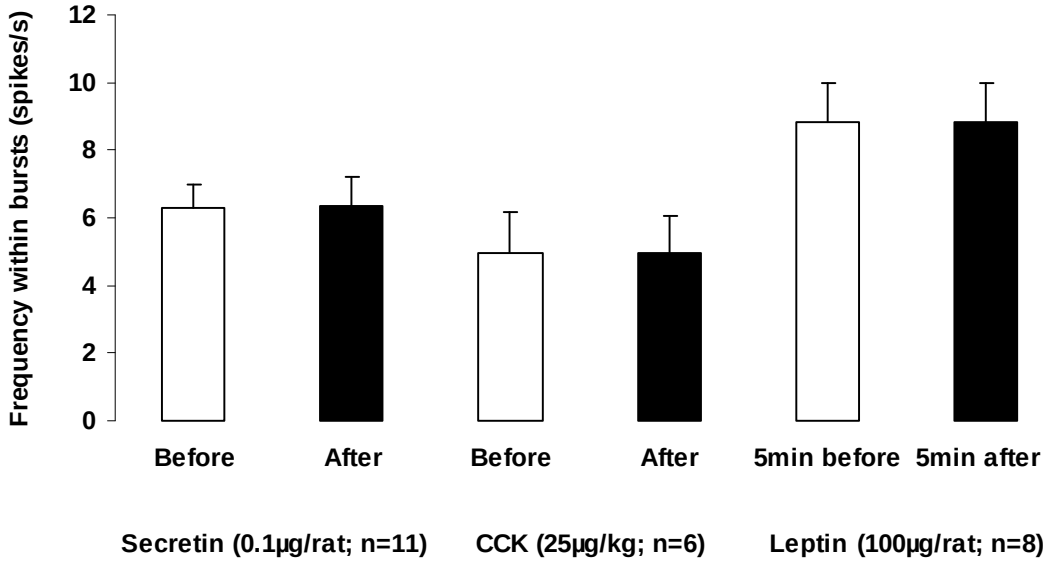


Fig. 6.32. Effect of secretin, leptin and CCK on phasic SON vasopressin neurones in unfasted virgin rats: Frequency within bursts (spikes/s). Values are mean ± s.e.m. Pre- *vs.* 0-5min post-secretin within the secretin group: P=0.3, paired t-test. Pre- *vs.* 0-5min post-CCK: P=0.4, paired t-test. Pre- *vs.* 0-5min post-leptin: P=0.9, paired t-test. The mean frequency within bursts during basal (p=0.06, Kruskal-Wallis one-way ANOVA on ranks) and 0-5min after administration of peptides (p=0.1, one-way ANOVA) did not differ between the groups. Secretin, CCK and leptin did not have any effect on the frequency within bursts of SON vasopressin neurones. n.s]

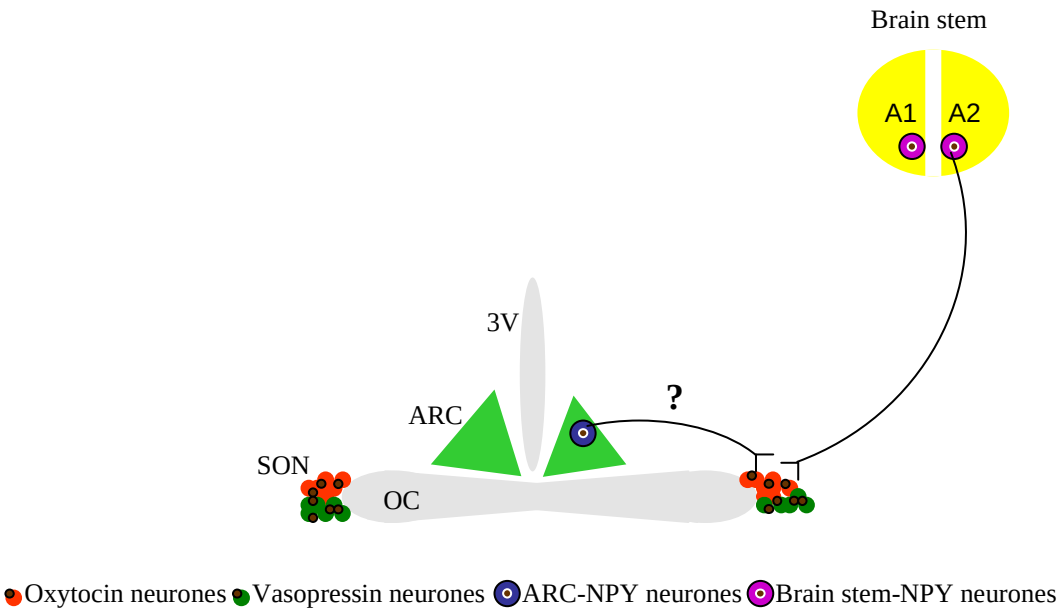


Fig. 7.1. Illustration showing hypothetical NPY neuronal projections to the SON. NPY is co-localized with NA in the A1 cell group but co-localization in A2 regions of the brainstem is controversial (Sawchenko and Swanson, 1982; Harfstrand et al., 1987; Simonian and Herbison, 1997). NA neurones from the A2 cell group project to the SON oxytocin neurones (Onaka et al., 1995). However it is not known whether these neurones co-express NPY. ARC NPY expression is upregulated prior to feeding or during food deprivation (Kalra and Kalra, 2003). Although ARC neurones project to the SON (Leng et al., 1988), it is not known whether ARC-NPY neurones project to the SON. NPY actions on SON oxytocin neurones may be indirect.

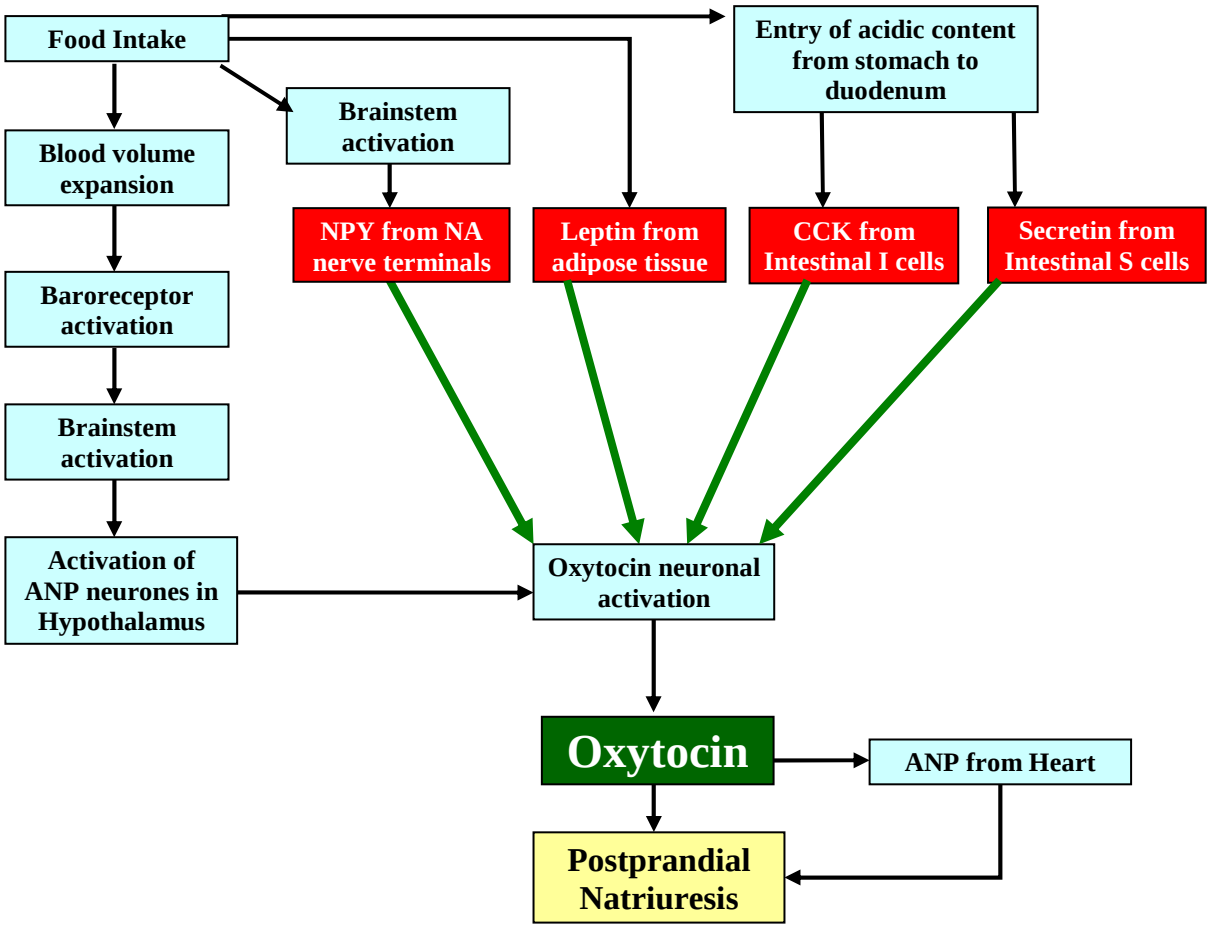


Fig. 7.2. Flow chart showing the peripheral and neuroendocrine events taking place around feeding that hypothetically culminate in oxytocin release so as to regulate postprandial natriuresis in the rat. Fluid intake accompanies food intake, resulting in blood volume expansion. Activation of baroreceptors leads to activation of neurones in the brainstem which results in the activation of ANP neurones in the hypothalamus. Further activation of oxytocin neurones leads to peripheral release of oxytocin. Oxytocin, by its direct action on the kidneys or through the release of ANP from the heart, mediates postprandial natriuresis (Haanwinckel et al., 1995). In addition to blood volume expansion, oxytocin neurones are also activated in response to other peptides that are released around feeding (green arrows), as found (or, for CCK, confirmed) in this study. Food intake is followed by transient increase in leptin mRNA in the adipose tissue and increased plasma level of leptin during the postprandial period (Saladin et al., 1995; Konturek et al., 2003). The passage of acidic chyme from the stomach to the duodenum stimulates the release of CCK and secretin from the duodenum which subsequently act on the exocrine pancreas to increase bicarbonate and enzyme rich secretion, respectively, to facilitate digestion (Folsch and Wormsley, 1973). Food intake activates brainstem neurones via vagal afferents (Rinaman et al., 1998) which might result in NPY release from NA terminals of the axons projecting from the brainstem to the SON. This study showed that NPY, leptin and secretin excite SON oxytocin neurones. CCK-induced excitation of oxytocin neurones is well established (Renaud et al., 1987). It is tempting to propose that NPY, leptin and secretin, along with CCK, also contribute to postprandial natriuresis via oxytocin release.

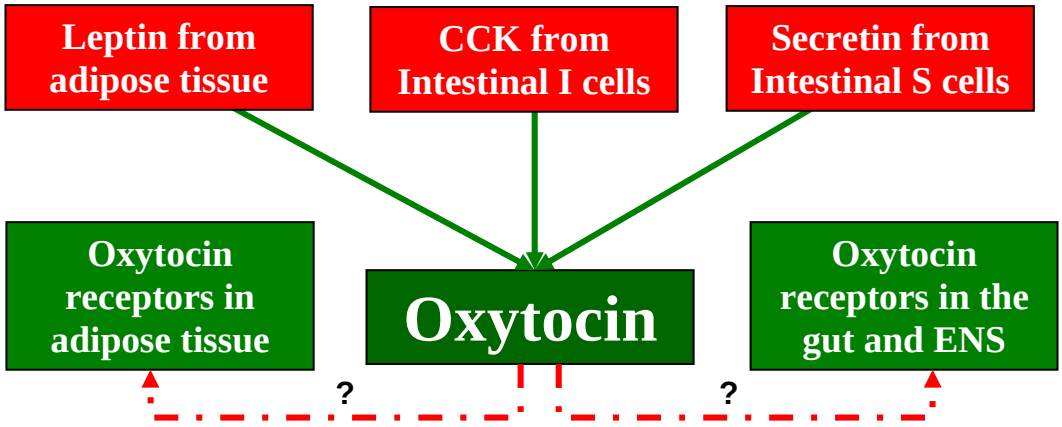


Fig. 7.3. A flow chart describing the possibility of reciprocal regulation of leptin/CCK/secretin by peripheral oxytocin. This study shows that leptin, CCK and secretin activate SON oxytocin neurones. As oxytocin receptors are expressed in the adipose tissue, gut and enteric nervous system[13] (ENS) (Bonne and Cohen, 1975; Egan et al., 1990; Welch et al., 2009), it is possible that leptin-, CCK- or secretin-induced release of oxytocin feedback to the sources of these peptides to positively or negatively regulate their synthesis/release. Oxytocin might also modulate effects of secretin indirectly: high doses of ANP and CNP attenuate secretin-induced pancreatic secretion via a PLC/PKC pathway while lower doses increase this secretion (Sabbatini, 2009).

[13] Enteric nervous system (ENS): It is the intrinsic innervation of the gastro-intestinal tract located between the external muscle layers of the gut wall. It is composed of neurones and glial cells arranged in interconnecting ganglia. Within the ENS, the neurones vary in their morphology and functions. Many neurotransmitters expressed in the CNS are expressed within subclasses of neurones in the ENS. The functions of the ENS are: regulating gut motility reflexes, co-ordinating secretion and absorption, controlling blood flow and modulating immune and endocrine functions. The ENS is capable of mediating reflex activity in the absence of input from the brain or spinal cord. The presence of local motor circuits (sensory neurones, intrinsic primary afferent neurones, inter-neurones, and excitatory and inhibitory motor neurones) is responsible for this independent activity. [Burns AJ, Thapar N (2006) Advances in ontogeny of the enteric nervous system. Neurogastroenterol Motil 18:876-887.

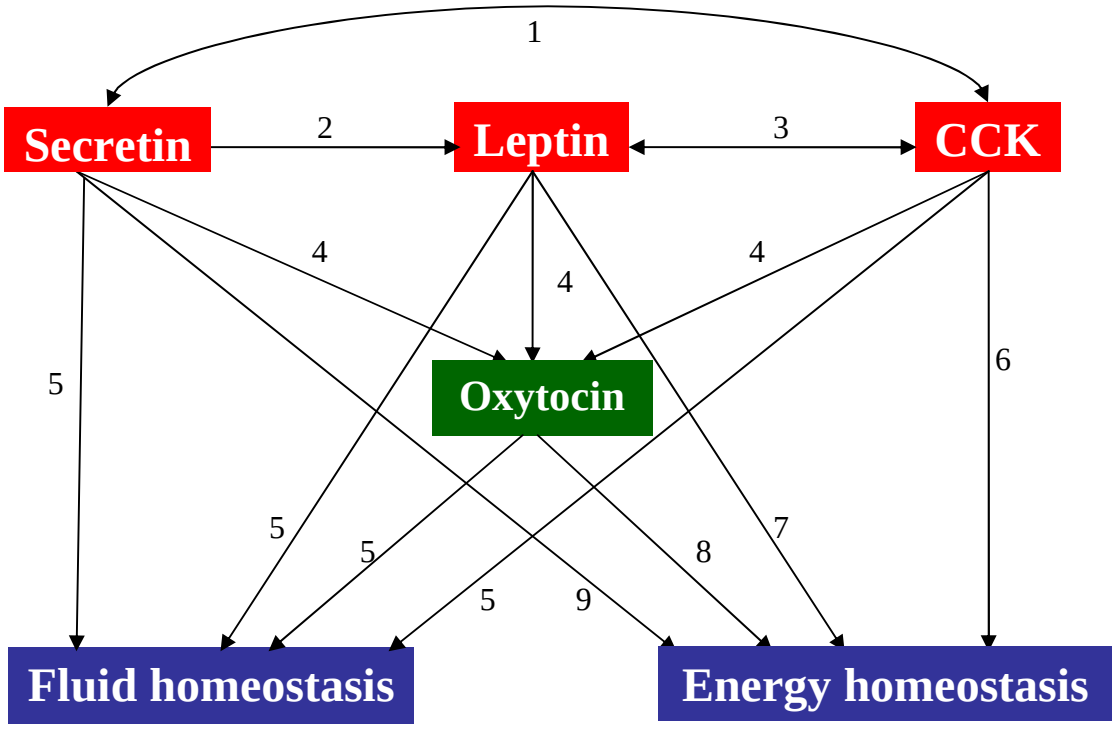
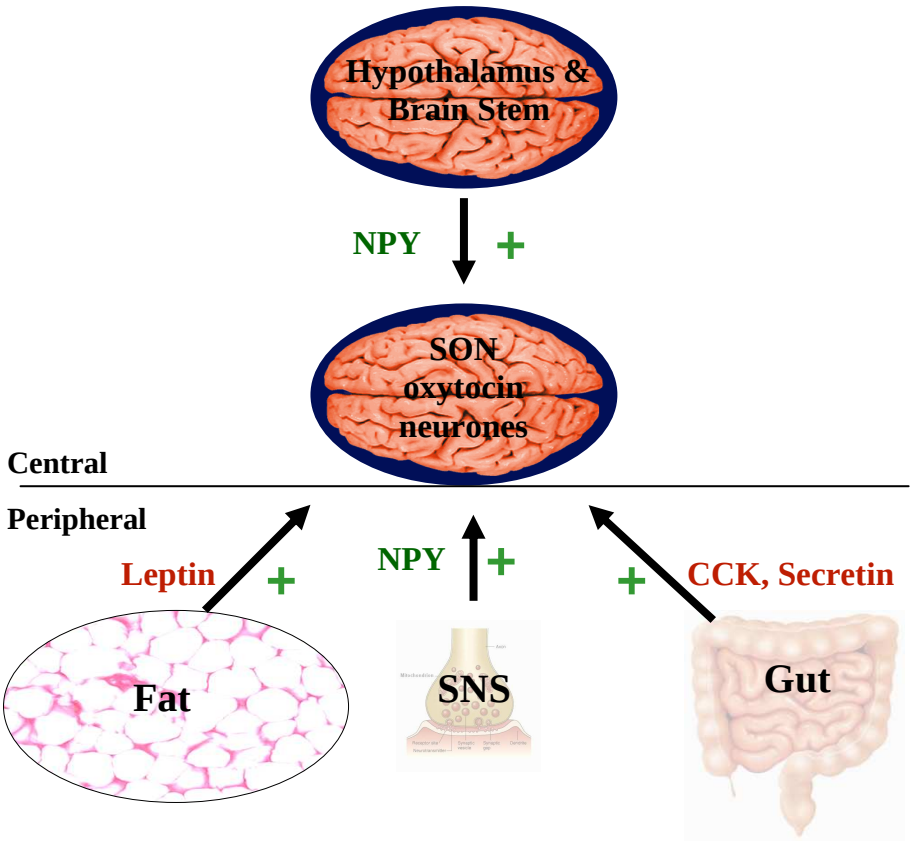


Fig. 7.4. Flow chart showing the proposed involvement of leptin, secretin and CCK in fluid and energy homeostasis directly and via the activation of SON oxytocin neurones. (1) Secretin and CCK are synergistic with each other in stimulating exocrine pancreatic secretion (Chey *et al.*, 1984); (2) Secretin increases circulating leptin levels (Sobhani et al., 2000); (3) Leptin is synergistic with CCK in food intake and body weight regulation (Barrachina et al., 1997; Wang et al., 1998); (4) This study shows that secretin, leptin and CCK excite SON oxytocin neurones [but leptin and CCK did not interact]. Increase in the electrical activity of oxytocin neurones results in the peripheral release of oxytocin; (5) Secretin, leptin and oxytocin are natriuretic either directly or indirectly (Waldum et al., 1980a; Waldum et al., 1980b; Waldum et al., 1981; Haanwinckel et al., 1995; Jackson and Li, 1997; Villarreal et al., 1998; Jackson and Herzer, 1999); CCK influences postprandial natriuresis via oxytocin (Verbalis et al., 1986; Verbalis et al., 1991); (6) CCK increases exocrine pancreatic enzyme secretion for digestion of carbohydrates, proteins and fats; CCK is also involved in the regulation of food intake and satiety (Gibbs et al., 1973); (7) Leptin negatively modulates exocrine pancreatic secretion (Konturek et al., 2003); is directly involved in the regulation of food intake, body weight and energy expenditure, i.e. energy homeostasis (Friedman, 1998); (8) Oxytocin regulates exocrine pancreatic secretion (Bjorkstrand et al., 1996; Ferrer et al., 2000). Central oxytocin is anorectic (Olson et al., 1991); (9) Secretin increases exocrine pancreatic secretin mainly of bicarbonate and water to neutralise the acidity so as to provide a neutral/alkaline medium for the digestive enzymes to act on carbohydrates, proteins and fats (Bayliss and Starling, 1902). Secretin has not been shown to modify food intake in rats (Lorenz et al., 1979; Garlicki et al., 1990). In summary, secretin, CCK and leptin are involved in fluid and energy homeostatic mechanisms either directly or indirectly through oxytocin.



- **Central NPY and systemic leptin, secretin, CCK and NPY: Excited SON oxytocin neurones**
- **Secretin: Dose-dependent increase in oxytocin neuronal activity and peripheral release of oxytocin**
- **On oxytocin neurones: Secretin >CCK > leptin**
- **Secretin-induced effects in oxytocin and vasopressin neurones: via NA pathway**
- **Responses in fasted rats: No difference from unfasted rats**
- **Pregnancy: Oxytocin neurone response to NPY and leptin did not differ from virgins**
- **Leptin and CCK Interaction: No potentiation by leptin of CCK-induced oxytocin neurone excitation**
- **Systemic NPY: Excited SON oxytocin neurones**
- **On vasopressin neurones: Variable effects (central NPY: ineffective; systemic NPY: inhibition; CCK: predominant inhibition; leptin: no significant effect; secretin: predominant excitation).**

Fig. 7.5. Thesis conclusions. The study was aimed at testing and comparing the actions of well-established or putative appetite regulating peptides, namely NPY, leptin, secretin and CCK, physiologically originating from the brain (NPY) or from the periphery [adipose tissue (leptin), sympathetic nervous system (SNS; NPY) and the gut (CCK and secretin)] on the SON oxytocin neurones in anaesthetised female rats (virgin or pregnant, fasted or not). All these peptides influenced the firing of oxytocin neurones. Other important results are summarised.